Reproduction and environment

Collective Expert Report

Synthesis

Inserm

French National Institute for Health and Medical Research

This document presents the synthesis made by the group of experts brought together by Inserm under the collective expertise procedure (appendix 1) to answer the request of the French Ministry of Health regarding chemical substances accessible to the general public and their effects on reproduction.

This work is based on the scientific data available during the second half of 2010. The expert report is based on the information contained in almost 1,700 documents.

Inserm's Collective Expertise Center, affiliated to the Public Health Thematic Multi-Organization Institute, coordinated this collective expertise.

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Preface

The remarkable collective expert report drawn up on endocrine disruptors (EDs) under the auspices of Inserm emerges as the most exhaustive synthesis of knowledge in the field to date. It paints an analytical and critical picture of the present situation that will certainly provide food for thought and help with decision-making in a very sensitive field as reminded by the news at regular intervals.

The five chemical substances or families of chemical substances (bisphenol A, phthalates, flame retardants, perfluorinated compounds and parabens) analyzed in this collective expert report have already been present in the environment of Western populations for several decades. They can be found in biological liquids (blood, urine, amniotic fluid, breast milk, etc.) and tissues in men, women, children and even fetuses. Regarding endocrine disruptors, their potential effects on the human reproductive organs and function are a concern legitimately raised by the public authorities. The effects reported – sometimes in humans but usually in experimental studies on animals – are difficult to link to the mechanisms of action currently known. It is therefore evident that endocrine disruptors bring into play the physiological mechanisms of signaling, regulation and action rather than the conventional mechanisms of toxicity leading to malfunction or cell death. Their study requires looking into the complexity of endocrine regulations and mechanisms of development, particularly during the critical stages of development, during embryonic and fetal life.

One of the key lessons of the collective expert report is that the emergence of the endocrine disruption problem sanctions the return to an integrative view of the living organism, i.e. consideration of the physiological and environmental complexity. Toxicology is thus going back to its roots since some of the scientists who built modern biology and experimental toxicology in the 19th century were also such eminent physiologists as Claude Bernard and François Magendie. Since then, anatomic pathology, diverse analytical methods, the study of genotoxicity and, more recently, molecular and cellular biology and multivariate approaches have considerably enriched the field of experimental toxicology and epidemiology.

In the preface of this report we are able to broach the beginnings of a new scientific approach for studying endocrine disruptors that would take on board the actual exposure of populations to a series of chemical substances which potentially have the same targets.

Traditionally, the toxicological approach has sought to assess the potential effects of a given molecule on different biological systems in vivo, ex vivo or in vitro. Beyond the intrinsic complexity of the endocrine disruption concept, an additional degree in this complexity appears with the notion of mixtures, i.e. cocktails of toxic substances that may – at cell, organism and even population and ecosystem level – have additive or synergistic effects according to Hass et al. (2007) and Christiansen et al. (2009), or effects described as "something from nothing" by Kortenkamp (2007, 2008).

This situation prompts toxicology to go further in its approaches, "expology" (analytical chemistry) to develop its technologies and epidemiology to factor in the multiple concomitant types of exposure in causal models by considering them in a combined rather than isolated manner, as is the norm in causal chain studies. The concepts of "multicausality" and causal networks now need to be referred to. Living organisms are always exposed to a multitude of environmental compounds that are toxic in nature. These compounds interact between them; their effects also depend on people's characteristics (their genotype for example) and on other behavioral and environmental types of exposure. The major challenge for the decades to come is to be able to decipher the composition and actions of these mixtures and to identify the most toxic molecules as well as the different ways in which their effects interact. This concerns an unquestionable public health problem aimed at getting toxicologists, biologists and epidemiologists working more closely together in the real world and at providing intervention tools.

In international research, consideration of the notion of mixture has resulted in the recent development of the concept of "exposome". As such, in the future each person will be able to access their "exposure ID card" from birth to adulthood thanks to exposomic technologies, just as they will be able to see their "genetic ID card" thanks to genomic technologies. In practice, the question of mixtures is one for both public decision-makers and researchers. Let's take a very simple example: let's say that a foodstuff is contaminated by some ten EDs with identical targets and similar methods of action. Let's also admit that exposure to each of these EDs is just below the threshold limit value (TLV). Remember that these TLVs are calculated for each compound. If exposure to this compound is assessed as being below the threshold determined, it is admitted to be "safe" and the foodstuff may be consumed with no apparent concern. If the ten EDs are all below the thresholds, they are each considered to have no effect and, all together, safe (ten times nothing is still nothing!). However, we could also take a completely different approach. Suppose that the method of action of the ten EDs is similar, it is then possible to calculate an equivalent dose by factoring in the real dose of each ED and its "efficacy" at triggering the toxic effect. By adding together the equivalent doses of EDs in the initial mixture, the threshold values will be vastly exceeded and the foodstuff may no longer be considered truly safe. In this approach, the additivity of equivalent doses is taken into account. Several recent studies on diverse EDs highlight the relevance of this type of approach (Kortenkamp, 2007, 2008).

Moreover, the possible manifestation of such effects will depend on the characteristics of the people exposed, their own sensitivity to the substances in question and their other risk factors. There is now no turning back from integrative approaches, as they are the only ones to be able to guarantee the significant headway expected by society as regards the Health-Environment interface.

There is a famous precedent for the notion of equivalent dose, since it concerns dioxins and "dioxin-like" compounds such as furans and some PCBs. In this case, we

knew that we had to add together the equivalent doses (TEQ),[1] hence why regulations were put in place. It seems logical to take an equivalent dose approach for EDs with the same mechanisms. That said, more basic research shows that, for dioxins, these notions still need adjusting as the nature of the toxin does not only dictate its efficacy but can also qualitatively alter the type of end effect – even when these toxins affect the same receptor. The most difficult combined effects to explore are not those concerning mixtures of products with the same mechanism of action, but those of products that bring different mechanisms into play. In this case, the effects can simply be additive, synergistic or even antagonistic in theory. Some EDs have agonistic-type activities towards estrogen receptors, while others are fairly effective antagonists. The result is not easy to determine in the event of a very complex mixture. On the other hand, some estradiol agonists can be associated with androgen receptor antagonists, in which case a potentialization of end effects may be observed. Mixtures can also have more subtle effects, for example when one of the toxins interferes with the metabolism or kinetics of one of the compounds present or disrupts the adaptive systems activated by another compound.

There are a great many examples of this type in the drug field, and they can also be found for contaminants (Ambolet-Camoit et al., 2010). In these situations, the usual criteria for causality set by Bradford Hill (strength of association, dose-response relationship, specificity) are not enough. They have to be considered from the viewpoint of multicausality or causal networks. Accordingly, taken alone, none of the substances may be held responsible for the occurrence of adverse effects, whereas their mixture will be. These research questions will have major consequences in risk analysis. We may well feel somewhat discouraged upon examining the degree of complexity that the study of contaminant mixtures represents. How on earth can the interaction of a hundred, a thousand pollutants be modeled? A possible response is provided by an American program called Tox21, which follows on from the National Academy of Sciences report entitled "Toxicity testing in the twenty-first century: a vision and a strategy".[2] In a nutshell, this program sets out to identify simple tests for the several dozen most represented toxicity pathways and to deliberate about one or more of these toxicity pathways rather than about a given product. The advantage of this approach is that it narrows down the study from all chemicals (over a hundred thousand) to all the pathways activated (twenty or so). The Tox21 project initially aims to test a few thousand contaminants and determine the main pathways that are activated by these substances. After this systematic work, methods for modeling the interactions between the pathways identified from mechanistic knowledge and systems biology findings must be found before these results are tested in a real-life situation. Understanding the mechanisms in play in detail and obtaining formal proof of causality in humans is a huge undertaking that will be impossible to complete any time soon.

[1] The "Toxic Equivalents" (TEQ) system expresses the relative toxicity of each less toxic compound as a fraction of the toxicity of the most toxic, TCDD. A "Toxic Equivalency Factor" (TEF) is attributed to each compound. This weighting coefficient indicates the degree of toxicity compared with the 2,3,7,8-TCDD, to which a reference value of 1 has been given.

[2] http://www.nap.edu/catalog/11970.html

But for all that, although these objectives remain essential and of primary importance, that does not mean courses of action cannot be considered right now. This is because the type of modeling described above makes it possible to identify possibilities of potential blocking of causal chains and interactions, without requiring a grasp of the exact arrangement. It should be possible to apply the precautionary principle as soon as suspicions founded on scientific data call for action to eliminate or reduce serious or irreversible health effects due to non-compulsory exposure.

Even though the scientific complexity, degree of uncertainty or ignorance prevent an understanding of all the mechanisms of action, protecting population health and setting about producing substitute substances should not be put on hold until proof of the causality and an understanding of these mechanisms have been obtained.

The decisions to be taken are difficult and will have significant consequences; they need to be reviewed as and when scientific breakthroughs are made. Formal consultations involving all of the stakeholders (comparable to the Intergovernmental Panel on Climate Change/IPCC for example) may be undertaken on these subjects with a view to making collective decisions under the auspices of the United Nations (UN) and/or World Health Organization (WHO).

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Foreword

Many genetic, behavioral and environmental factors are likely to have an effect on fertility or the development of the reproductive system[3]. Exposure to chemicals – particularly endocrine disruptors that are widespread in the environment – is an important type of risk to explore since it could be controlled.

The European regulations lay down bans and restrictions regarding the marketing and use for the general public of chemicals that are recognized as category 1[4] or 2[5] reproductive toxins. A certain number of chemical substances that are neither carcinogenic nor mutagenic are currently classed as category 3 reproductive toxins.[6] For the latter, effects on the reproductive function are possible, but there is insufficient proof for placing in category 2.

Over recent years, publications from various independent research laboratories have drawn attention to possible effects that chemical substances found in consumer products have on the reproductive function or organs. Studies usually conducted in vitro or in different animal species, sometimes epidemiological studies, have sounded the alarm for public authorities and health agencies.

In the light of society's questions over the possible risk posed by chemical substances accessible to the general public, the French Ministry of Health called on Inserm to perform an analysis of the data available on the effects of some of these substances on reproduction. To respond to the Ministry's request, a multidisciplinary group of experts made up of epidemiologists, chemists, endocrinologists and biologists specializing in reproduction, development and molecular genetics began by reviewing current knowledge in reproductive biology, physiology and epidemiology as well as the methods of research into the health impact of environmental contaminants. For each of the families of chemical substances in question, the literature analysis was then organized around the following questions:

• What are the main sources and pathways of exposure for the general population to the substance in question?

• What data is there on the impregnation of populations according to gender, age and other criteria? Can the most exposed populations be identified?

• What are the toxicokinetics of the compound and its metabolites in the body?

• What do the epidemiological studies having linked a case of exposure to the substance in question with the onset of reproductive function problems indicate?

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3 Jégou B, Jouannet P, Spira A. La fertilité est-elle en danger? Éditions La Découverte, Paris, 2009: 231p

4 Substances known to alter fertility in humans and/or to trigger toxic effects in the development of humans (risk phrase: R60 or R61)

5 Substances that should be compared to substances that alter human fertility and/or cause toxic effects on the development of humans (risk phrase: R60 or R61)

6 Worrying substances for fertility in humans and/or for humans because of possible toxic effects on development (risk phrase: R62 or R63)

• What are the effects observed in the experimental studies conducted in rodents, other species of mammal or species considered to be sensitive such as aquatic vertebrates, at doses compatible with the exposure levels in humans?

• What are the effects observed in animals depending on the exposure periods (prenatal, neonatal, prepubescent, postpubescent)? Can critical exposure periods be defined?

• What are the effects specifically observed in the tissues of the male and female reproductive system in in vivo studies from (possibly human) cell culture?

• Which mechanisms can be mentioned to explain the effects? Is the expression of certain genes altered? Are these alterations passed on to the next generations?

• Can data available on the structure-function relations shed light on the toxicity of substances from the same family?

• Which lines of research should be given precedence to go further in the study of risks potentially associated with chronic multi-exposure?

Five substances or families of substances have been chosen because they are widely represented in consumer products: bisphenol A, phthalates, polybrominated compounds or flame retardants, perfluorinated compounds and parabens. The health events considered in relation with exposure to these substances are the quantitative and qualitative characteristics of sperm and of fertility (biological aptitude to fall pregnant), all the abnormalities of the organs involved in the reproductive function (gonads, mammary gland, endocrine system), testicular cancer and certain so-called hormone-dependent cancers. Although developmental problems affecting other organs and diverse diseases may, in some cases, be brought about by exposure during intrauterine life, they are not addressed in this expert report. A table presented in Appendix 2 brings together all of the main data on each of the 5 substances or families of substances. The difficulty in characterizing the risk associated with human exposure to each of these chemical substances is even greater when you consider that most of the population (including pregnant women and fetuses) is exposed to a series of substances likely to have very different methods of action. Studying the effects of combined, ongoing exposure to ubiquitous substances in the human environment is a crucial research challenge for the purposes of better risk management.

Synthesis

According to a certain number of studies, an increase in the prevalence of problems affecting the male reproductive function has been observed in several Western countries over the last few decades. The best documented data concerns testicular cancer.

It has been clearly demonstrated that the incidence of this cancer – the most common in young men – has risen over the last 50 years in many European countries. This increase is to the tune of 0.1 to 0.2 cases for 100,000 people-year, leading to a doubling in the incidence in European countries since 1970. In France, there has been an average rise of 2.5% per year over the 1980-2005 period. In 2010, the incidence rate in France was estimated to be 7 cases for 100,000 people-year. This increase cannot be explained by either population aging or an improvement in screening practices.

Two types of relatively common malformations – hypospadias (abnormality of the external male genital tracts) and cryptorchidism (abnormality of the testicular descent observed at birth) – also seem to be on the rise; register data is less reliable than for testicular cancer however, and there are few one-off studies. Major geographical variations can be observed. The data available in France shows a clear increase in the incidence of hypospadias since the late 1970s up until the early 2000s. For cryptorchidism, there is not enough data in France to estimate its development.

At the same time, a fall in sperm count has been reported in North America and Europe. In France, the studies conducted using data from French Human Egg and Sperm Bank and Research Centers (CECOS) also indicate a significant drop in sperm count as well as in morphologically normal sperm motility in some regions. Overall, the temporal deterioration in several sperm characteristics can be considered plausible in some industrialized countries.

Knowledge of temporal changes in couple fertility is much more limited. The few studies that are based on such indicators as fertility (estimated on the basis of the time needed to conceive) and which often had methodological limitations do not show any alteration over time in the fertility of couples in certain regions of Sweden, Denmark and the UK. Indirect studies indicate that a deterioration in sperm characteristics could have had an impact on the proportion of couples suffering from involuntary childlessness or who are eligible for assisted reproductive technology. Overall, it is not possible from these studies to draw any strong conclusions as to the temporal change in couple fertility over the last few decades in industrialized countries. With no fertility surveillance system in most of these countries, it is unlikely that a response to this question will be forthcoming any time soon.

At the beginning of the new millennium, Professor Skakkebaek's team in Copenhagen came up with a hypothesis that the occurrence of testicular cancer, alteration in sperm quality and production, cryptorchidism and hypospadias could all have been caused by a disruption in testicular development during fetal life. The concept of "testicular dysgenesis syndrome" that has been put forward remains controversial however.

In girls in Western countries, the most striking observation is the longstanding trend towards earlier puberty. The curve of this trend varies between countries. According to Norwegian, Finnish and American findings, an onset 0.3 years earlier per decade has been estimated; in France, an onset 0.18 years earlier per decade has been observed.

With no historical data on environmental exposure at population level, it is not possible from descriptive studies on temporal changes in the reproductive function to explore the causes of these changes. Many etiological studies have been conducted to try and explain the differences observed between individuals; but they can only provide indirect information about these variations. At present, the data suggesting a possible impact on the reproductive function is most complete for such factors as smoking (at adult age or passively during intrauterine life), for certain occupational exposure and for the most persistent pollutants in the body. To analyze the impact of exposure to chemical compounds on reproductive health, many studies in animal toxicology and epidemiology have been performed in different risk assessment or research contexts. To transpose research findings from one species to another, knowledge is required about the similarities and differences during the different developmental stages of the reproductive function.

Reproductive function and differences between species

In all vertebrates, the testis and the ovary develop from an embryonic primordium that is initially bipotential male and female. At different moments of its development (which vary depending on species), this primordium evolves towards a male or female differentiation according to its genetic heritage (mammals, birds) or such environmental factors as temperature or behavior (some reptiles and fish). A distinction can therefore be drawn between a genetic determination of gender and environmental determination. It is important to take these different methods of sexual differentiation into account when assessing the effects of environmental factors like endocrine disruptors. An alteration in environment will have more consequences on fish and reptiles than on other species of vertebrate.

In mammals, gender is genetically determined at fertilization depending on whether or not the sperm carries the Y chromosome. The SRY gene on the Y chromosome brings about the male differentiation of the gonad's somatic cells into Sertoli cells. In females, with no Y chromosome, the differentiation of the ovary occurs from the bipotential gonad. The fetal and neonatal development of gonads is a particularly sensitive phase and any chemical substance able to disrupt these early stages will have repercussions on the reproductive function.

Depending on gender, gonads differentiate to produce gametes (gametogenesis of germ cells) and synthesize hormones (steroidogenesis) under the effect of which the internal and external genital tract will evolve to enable reproduction: development of a reproductive system in keeping with the sex of the gonad which will carry out the functions of gamete maturation, insemination, fertilization or parturition.

In males, steroidogenesis begins during fetal life in all species, whereas in females the stage at which it gets under way depends on the species. Accordingly, species with an active hormone system during fetal life (ruminants, humans) may be more sensitive to the effects of endocrine disruptors for example. Very early on, the fetal testis secretes two types of hormone: the anti-Mullërian hormone secreted by the Sertoli cells and testosterone secreted by the Leydig cells. Testosterone is responsible for the differentiation of male genital tracts. The protein Insulin-like 3 (INSL3), produced by the fetal and adult Leydig cells, is responsible for the development of the gubernaculum involved in testis descent.

In mammals, gonads form during intrauterine life over the first third of gestation. There is a time lag between male and female differentiation. Testicular differentiation occurs earlier than ovarian differentiation. This implies that exposure to an endocrine disruptor at a given moment of *in utero* development will not have the same effects on male and female fetuses.

Differentiation of the male genital tract depends much more on the production of hormones than the female's does. This is because the fetus's ovaries are not essential for the feminization of the body, whereas testis are for masculinization. This needs to be borne in mind if we are to understand why some substances bring about more marked effects in males than in females. Any disturbance in early hormonal function will certainly have more marked effects on males than females.

However, the sudden reduction or disappearance of a large number of germ cells will have repercussions on the differentiation of the ovary, when the lack of spermatogonia does not influence testicular differentiation.

The reserve of primordial follicles formed in the fetal ovary is fixed and determined for the whole of the female's reproductive life, whereas spermatogenesis produces gametes continuously – from puberty through to senescence – in males. Any quantitative alteration in germ cells occurring very early on in females will therefore be irreversible and may have very long-term effects (20 to 30 years) on fertility. In males, qualitative alterations risk having long-term effects instead.

The different stages of ovarian and testicular development, although similar overall in all mammals, present significant variations between species and do not take place over similar periods. For example, follicular formation begins in the ovary during fetal life in humans and during postnatal life in rodents. And yet rodents are usually used as models in reproductive toxicology.

There are also physiological (and pathological) differences with regard to the reproductive function and development between mammals which sometimes make difficult extrapolation between rodents and humans. As such, vaginal opening, which is the beginning of puberty in mice, does not exist in primates. On the other hand, endometriosis is a disease specific to primates.

What's more, rodents are polyovular animals: at each cycle, a dozen or more follicles will reach ovulation and a dozen ovocytes will be produced. The mechanisms regulating folliculogenesis and ovulation are therefore different in rats or mice and in monoovular species like humans and ruminants.

Rodents are born much more immature than most other mammals, which limits extrapolation of exposure received after birth. The neonatal period of rodents corresponds, from a developmental point of view, to the end of pregnancy in humans. As a result, the period in which ovarian folliculogenesis or the development of the central nervous system takes place differs between humans and rats such that disruptions during gestation will have more impact on both of these mechanisms in humans than in rodents.

In humans, the testis, just like all the endocrine glands, is controlled by the hypothalamo-hypophyseal complex. Gonadotrophin (GnRH) released by the hypothalamus stimulates the secretion of two hypophyseal hormones: follicle stimulating hormone (FSH) and luteinizing hormone (LH). Acting on Sertoli cells, FSH plays a role in initiating spermatogenesis. At puberty, LH increases testosterone production, which acts directly on Sertoli cells to ensure spermatogenesis takes place properly.

In women, the ovaries secrete two hormones, estradiol and progesterone. During folliculogenesis, granulosa cells (which come about in the same way as Sertoli cells) become sensitive to FSH and will continue to multiply and differentiate (like Leydig cells in males). Androgens secreted diffuse in granulosa cells and, under the influence of the FSH, are turned into estradiol. The differentiation of granulosa cells also produces follicular liquid and the follicle becomes the antral follicle. The sudden secretion of LH triggers the final maturation of the ovocyte and ovulation.

Caution must be taken when extrapolating results obtained in rodents (mice, rats) to humans and the chronological and physiological differences existing between the two species must be taken into account. Other animals appear to be more relevant than rodents, particularly those that present long gestation and life periods, are monoovular and only carry one baby per pregnancy, such as ruminants.

The effects of in utero exposure to chemical substances can manifest over the very long term, or sometimes lessen over time. It is therefore vital to conduct longitudinal studies with several investigation points (at birth, during weaning, in puberty and adulthood). The time interval between the moment exposure ended and the study must be factored in as it may explain part of the contradictory findings of the scientific literature.

Metabolism

The fate of a xenobiotic in the body can be illustrated according to four major stages: absorption; distribution (with possible storage in the target tissues or organs); biotransformation of the substance absorbed; elimination.

Absorption first of all depends on the physicochemical properties of the substance itself, namely its molecular mass, degree of ionization, reactiveness and solubility. Lipophilic chemicals are the most capable of crossing a membrane whose constituents are lipids for the most part. That said, particularly within the intestine, very lipophilic substances are not absorbed as well because of the difficulty in forming a solution or emulsion in the intestinal lumen. The relationship between the

external and internal dose therefore largely depends on the level of absorption, which may itself be affected by the lipophilic nature of the substance or, for some compounds, the effectiveness of such efflux pump systems as P-glycoproteins.

The difference between oral absorption (i.e. presence in the intestinal wall and portal vein) and bioavailability (i.e. presence in the systemic blood and tissues) can, amongst other factors, stem from the chemical breakdown linked to the metabolism in the intestinal wall, from the efflux to the intestinal lumen or from the first-pass effect in the liver.

Although some membrane barriers are less permeable than others, the diffusion mechanisms generally follow the same rules as those that govern absorption and initially depend on the physicochemical characteristics of the xenobiotic. Crossing of the placental barrier should be studied closely as fetal life is a particularly sensitive period of development. Although fetal exposure to maternal estrogens is limited because of their binding to the α-fetoprotein (which is normally only produced by the fetus during its development), many chemical substances (labeled as endocrine disruptors) have much less affinity with this protein and therefore get into the fetal circulation easily. This is what seems to happen with bisphenol A.

In order to be excreted in the urine or bile, molecules must be soluble in water. The chemical transformation of the xenobiotic is mainly catalyzed by enzymes that function with endogenous co-factors. Conceptually, this process has been separated into two phases during which the xenobiotic is oxidized, reduced or hydrolyzed (phase I) and/or conjugated with glucuronic acid, a sulfate or acetate group, glutathione or an amino acid (phase II). These reactions not only involve xenobiotics but also endogenous compounds like steroids, prostaglandins and some vitamins.

Cytochrome P450 (CYP) monooxygenases, membrane enzymes situated in the endoplasmic reticulum, plays a key role in xenobiotic metabolism. There are some sixty different CYPs in humans and a dozen or so of these are used in xenobiotic metabolism, of which 1A1/2, 2C9, 2C19, 2D6 and 3A4 are the most commonly involved forms. An induction of CYP19A1 (aromatase) can foster excessive production of estrogens and feminizing effects. A genetic polymorphism, common for forms 2C9, 2C19 and 2D6, is expressed in interindividual differences in susceptibility to the action of toxins.

The phase II reactions most commonly used by the body are glucuronidation and conjugation with glutathione and the sulfate group. Although a large number of tissues can express biotransformation enzymes, the liver is the main organ involved in metabolism. It is nevertheless possible that some particular isoforms are specifically expressed in tissue outside of the liver. This is, for example, the case with aromatase which, in adults, is expressed in the ovaries, placenta, fatty tissue, bone and, to a smaller extent, the testis, but not in the liver – when the expression levels are very high in the fetal liver.

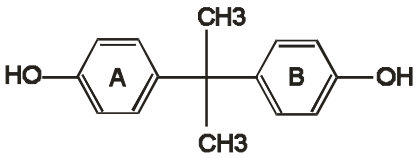
Bioactivation pathways usually involve production of a metabolite with a much stronger activity or affinity than the initial substance as regards a transport protein or nuclear receptor. Accordingly, substances that are inactive in their initial state can

become estrogenic after intervention of an enzyme, generally a CYP (this is the case for phthalates, polybrominated compounds and so on).

Once formed, metabolites are excreted in the urine by the kidney or eliminated in feces via bile. Substantial excretion in breast milk is also possible, as has been proven for polybrominated compounds (PBDE), phthalates and bisphenol A. In general, rodents excrete more metabolites through the biliary tract than dogs, monkeys or humans. This is due to differences between species in the biliary excretion threshold of metabolites. After being eliminated in bile, the sulfate and glucuronide conjugates can easily be hydrolyzed in the digestive tract. The biotransformation products released in this way are then reabsorbed by the intestine and once again metabolized in the liver. This is called enterohepatic circulation, the primary consequence of which is an increase in the time the xenobiotic stays in the body.

Bisphenol A

4,4-isopropylidenediphenol, more commonly referred to as bisphenol A (or BPA), is a synthetic chemical compound used particularly in the industrial manufacture of polycarbonate type plastics and epoxy resins.



Chemical structure of bisphenol A

Polycarbonates can be found in all sorts of everyday objects (CDs, glasses, some plastic bottles and baby bottles) and epoxy resins are found in the inside coats of food tins or dental amalgams. Bisphenol A is also used to make PVC and some plasticizers as well as heat-sensitive paper (issued, for example, by tills).

Bisphenol A is currently classed as a category 3 reproductive toxicant.[7] The risk assessments carried out at the request of international health agencies (EFSA in Europe) have resulted in the definition of a tolerable daily intake of 50 µg of bisphenol A per kg of body weight and per day, i.e. 2.5 mg per day for a person weighing 50 kg. Since July 2010, the manufacture, importation, exportation and marketing (for free or for a fee) of baby bottles made using bisphenol A have been suspended in France.[8]. The French Agency for Food, Environmental & Occupational Health & Safety (Anses) has recently actualized its opinion on Bisphenol A.

Over the last few years, an increasing number of studies conducted in academic research laboratories have documented diverse effects of bisphenol A on reproduction.

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[7] Category 3 reproductive toxicant: substance deemed "worrying for human fertility" because of "possible " but unproven toxic effects on reproduction

[8] French Act no. 2010-729 of June 30, 2010

Exposure

According to the international health agencies, the general population's main source of exposure is through food. It results from the transfer of bisphenol A into food or drink from plastic polymers or epoxy resins used to package or contain them.

In adults, some authors believe that the consumption of drinks in polycarbonate bottles, tinned foods or food heated in the microwave in their packaging leads to an average ingestion of 0.1 µg of bisphenol A per kg of body weight per day.

In its opinion dated January 2010, the French Food Safety Agency (Afssa), according to the literature data, put the exposure of infants from both baby bottles and the packaging of formula milk at between 0.2 and 2 µg of bisphenol A per kg of body weight per day. Similar data is presented in a recent report (Joint FAO/WHO Expert Meeting to Review Toxicological and Health Aspects of Bisphenol A Summary Report November 1-5, 2010).[9]

Handling heat-sensitive paper (issued by cash registers) or inhaling bisphenol A-contaminated dust could represent other sources of contamination particularly for some populations. Moreover, by-products of bisphenol A used as dental composites induce high saliva levels of bisphenol A in patients. This suggests that several exposure pathways or exposure to certain by-products of bisphenol A need to be considered.

Measurements of bisphenol A taken in the blood, urine, breast milk and other tissues indicate that over 90% of people living in Western countries are exposed to detectable levels of bisphenol A. Rates exceeding the detection limit of 0.5 µg/l have been found in the placenta, amniotic fluid and fetus in rodents and humans. Bisphenol A is therefore capable of crossing the placental barrier and reaching the fetus.

According to a German study, children (3-5 years old) constitute the sub-group with the highest impregnation, with a mean urine level of 3.5 µg/l. In France, the median value of total and free bisphenol A urine levels in a sample of women on the day they give birth are equal to 2.9 and 0.5 µg/g of creatinine respectively, and the free bisphenol A/total bisphenol A ratio is 0.17.

The exposure levels in adults and children alike estimated from urine levels correspond to an exposure below the tolerable daily intake of 50 µg/kg/d.

In adult men, bisphenol A absorbed by the digestive system is quickly eliminated in the urine in the form of BPA-glucuronide. The plasma half-life is around 4-6 hours. In the context of occasional exposure, most BPA is eliminated within 24 hours. Similar values have recently been reported in rodents and monkeys.

Extrapolations of pharmacokinetic data from animals to humans are complicated because of major inter-species differences in the elimination processes. Polymorphisms of conjugating enzymes in humans may trigger significant individual variations in the detoxification capacity. Lastly, deconjugating processes of metabolites (releasing bisphenol A) may occur in some target organs.

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[9] fttp://ftp.fao.org/ag/agn/agns/BPA_Summary_Report.pdf

Epidemiological studies

Few epidemiological studies have assessed – over the short or long-term – the effects of bisphenol A exposure on the reproductive function.

One study conducted in China between 2004 and 2008 has shown, in workers manufacturing bisphenol A-based products, that this exposure to BPA (around 50 times higher than in the general population) was associated with more sexual function problems being declared (erectile dysfunction or sexual dissatisfaction). A second publication by the same authors suggests that a link could exist in workers not exposed at their workstation between similar BPA levels to those of the general population and feelings of "sexual dissatisfaction".

In 2010, two studies on men seeking help for infertility or partners of pregnant women reported alterations in the levels of hormones involved in reproduction in association with higher urine levels of bisphenol A.

Two more studies have shown a reduction in sperm count in association with urine levels of bisphenol A corresponding to those found in the general population. Yet another study performed in fertile men found no association. No longitudinal study has been conducted to date on the matter and there is no human data on the impact of bisphenol A exposure during intrauterine life on the reproductive function in adulthood either. In all, very few studies have been carried out and it cannot be considered that bisphenol A, at the doses to which the general population is exposed, is safe for the reproductive function in men.

At present, studies conducted in women on the risk of breast cancer or endometriosis all take a retrospective approach (particularly limited for a non-persistent compound like bisphenol A) and are based on clinical populations for convenience, with no precise sampling plan, and are therefore not informative.

Studies in animals

The two main toxicity studies performed in rats and mice according to the Organization for Economic Co-operation and Development (OECD) guidelines did not reveal any significant effects on reproduction in males, females or their offspring, after bisphenol A exposure from gestation and over several generations, at similar doses to environmental exposure in humans (between 3 µg/kg/d and 300 mg/kg/d).

However, over recent years several studies conducted in academic research laboratories on different strains of rat and mouse and on the basis of diversified experimental protocols have highlighted effects that have not been studied much to date, and above all over specific exposure periods. These studies emphasize the consequences of bisphenol A exposure in utero and during breastfeeding that are likely to interfere directly with the development of the embryo and then the newborn, and to generate long-term effects on the young person's and adult's reproduction (male or female).

In male mice and rats, after exposure during gestation and the postnatal period, several studies reveal effects of bisphenol A on the genital system (testicular

hypotrophy, prostate hypertrophy, shorter anogenital distance, delayed separation of the foreskin, etc.), on sperm production, on the level of male hormones and on fertility (reduction in the size of litters) with doses of around µg/kg/d. These results have not been found in all the studies.

In female mice and rats, after exposure during gestation and the postnatal period, bisphenol A can bring on early puberty and alterations in the uterus, vagina, ovary and endometrium (effects appearing for doses ranging from 0.2 to 500 µg/kg/d according to the species and lines used).

Furthermore, after exposure in utero, abnormalities in maternal and sexual behavior are observed in both sexes.

The passing-down of some of these effects to the offspring of the rodents exposed suggests that bisphenol A can alter epigenetic information and disrupt genetic expression. These studies raise the problem of harmful effects being passed on to the next generations, and justify that longitudinal studies be undertaken over several generations.

Lastly, studies carried out on several lines of the same species of rodent show intra-species (and inter-individual) variabilities in response to bisphenol A. These variabilities reflect the genetic polymorphism and may also be attributed to prior exposure suffered during different stages of life. The fact that the alterations are not found in all the studies and demonstration of variable sensitivity of some lines cannot constitute arguments in favor of there being no effects; on the contrary they should prompt us to understand how genetic and environmental factors can alter responses to bisphenol A.

In aquatic vertebrates, BPA can alter the action of sexual hormones and trigger partial sex reversals as well as abnormalities in embryonic development at compatible doses with the amounts found in some rivers.

Target tissues and organs

Exposure to bisphenol A during the organ creation phase when gestation is taking place seems to be particularly critical.

For the female reproductive system, BPA exposure during the mammal tissue creation phase in utero can alter how this organ develops (at doses of 0.25 µg/kg/d), increase its sensitivity to estrogens during puberty and lead to the appearance of precancerous lesions (at doses of 25 or 250 µg/kg/d).

Likewise, the fetal or neonatal period seems to be a critical time during which bisphenol A exposure could alter prostate development and foster the appearance of precancerous lesions (with doses of 10 to 20 µg/kg/d).

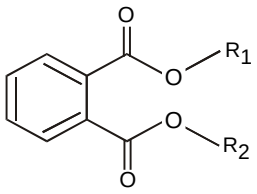
The onset of hormone-dependent cancers (breast or prostate) of the carcinoma type seems to be more likely following an alteration in the organ's development, caused by BPA.

The tumor risk would then be increased by exposure in adulthood to environmental carcinogens or hormones.

A link between bisphenol A exposure in utero and endometrial lesions (of the endometriosis type) is suspected.

Phthalates

Phthalates are the product of esterification of a phthalic acid with one or more alcohols. We can distinguish phthalic esters whose two acidic functions are esterified by the same alcohol (DEHP, DBP), different alcohols (BBP) or by oxo-type alcohols (DINP, DIDP).



Chemical structure of phthalates
R1,R2 =C2H5: DEP Diethyl-phthalate
R1,R2 =C8H17: DEHP Di(2-ethyl-hexyl)-phthalate
R1,R2 = C4H9: DBP Dibutyl-phthalate
R1,R2 = C9H20: DINP Diisononyl-phthalate
R1,R2 = C4H9, C7H7: BBP Butylbenzyl-phthalate

Some of the most commonly used phthalates, in addition to DEHP, are BBP, DBP, DEP and DINP.

Phthalates are found in various products used on an everyday basis such as adhesives, vinyl floor coverings, lubricants, electrical condensers, detergents, solvents, pharmaceutical products, electrical wires and cables and cosmetics (perfume, deodorants, shaving lotions, shampoos, hairspray, nail varnish and so on). What is particular about phthalates used in plastics is that they are not bound covalently to the polymers they endow with their flexibility. They can therefore easily migrate in packaging materials and be released in the environment, particularly when the plastics containing them are subject to high temperatures.

Bans and restrictions regarding their use have been introduced by the European Commission: in preparations for use by the general public (paints and glues, etc.), all CMR1 and 2 classified phthalates have been banned; concerning articles, DEHP, DBP and BBP are banned in the production of toys and articles for children; DINP, DIDP and DNOP (di-n-octyl phthalate) are banned in toys for children under three. Such phthalates as DBP, DEHP and BBP are not authorized in cosmetics; DEHP is also banned in food contact materials. The use of DEHP in medical devices is restricted if newborns, pregnant and breastfeeding women have to be exposed to them.

The risk assessments conducted by the health authorities (EFSA in Europe) on DEHP, DBP, DIDP, BBP and DINP have defined tolerable daily intakes of 50, 10, 150, 500 and 150 µg respectively per kg of body weight per day.

Exposure

Human exposure to some phthalates is high and rising steadily because of the widespread use of this family of compounds and the increase in production levels over the last thirty years.

This exposure can come from direct contact with air, water or food and results from inhalation, ingestion or percutaneous absorption of these products. The ingestion of food that has come into contact with packaging containing phthalates continues to be the main source of exposure for the adult general population. Food is, in particular, the main source of exposure to DEHP, DBP and DIBP (diisobutyl-phthalate). In children, ingestion via hand-mouth exposure may also be high.

DEHP exposure in adults is estimated on average to be around 2 µg/kg of body weight per day according to urine DEHP concentration or DEHP metabolite data in Western populations. It is slightly lower for other phthalates. Few differences are observed between men and women.

Compared (at the same time and in the same studies) to adults, all infants (0-3 years old) present urine concentrations 3 to 5 times higher. According to German data, 1.5% of infants in Germany present DEHP exposure exceeding the exposure level for which the absence of adverse effect is not certain. In France, in a sample of women on the day they give birth, the urine metabolite levels are, in median value, equal to 42µg/l and 28 µg/l respectively for 5OH-MEHP and 5oxo-MEHP.

Based on the DEHP content in baby food, the authors estimated that dietary exposure of newborns under 6 months old was around 10 µg/kg bw/d and almost 20 µg/kg bw/d in infants over 6 months old. Since the bans and restrictions regarding use, other phthalates (DINP) have been found more often, particularly in children.

Medical devices (blood bags, tubing, etc.) account for a fairly significant source of exposure to phthalates – particularly DEHP – for some sub-groups of the population. Exposure via medical devices mainly concerns hemodialysis patients, donors and recipients of platelets and premature babies. In 2008, the European Chemicals Bureau (ECB) estimated that exposure could reach 3,000 µg/kg bw/d in adult hemodialysis patients and 1,700 µg/kg bw/d in newborn babies undergoing transfusion.

According to the compounds, the plasma half-life in humans is 8 to 48 hours. It is 18 hours for DEHP. Although no bioaccumulation is observed, it is important to point out that, as with BPA, exposure is continuous because of the diversity of contamination sources (food, environment, cosmetics, etc.). Studies conducted in men or women show that some phthalates or their metabolites are found in urine, blood plasma, semen plasma, breast milk, amniotic fluid and umbilical cord blood.

Phthalate metabolism leads to the production of both oxidized and non-oxidized metabolites. Recent studies seem to indicate that, in humans, the main metabolites in urine are oxidized metabolites. Measuring oxidized metabolites in biological fluids excludes contamination associated with the equipment used during dosing.

The maximum blood MEHP (DEHP metabolite) concentration is 7.5 times lower in non-human primates (monkeys) than in rats. In humans and monkeys, MEHP is

found in the blood and urine mainly as a glucuroconjugate. However, DEHP metabolites, with carboxylate ester chains, are found in conjugated and free forms in human urine samples. Different tissues can be the target of these metabolites (testis, ovary, etc.). The differences in absorption, distribution, metabolism and excretion between species (rodents, monkeys and humans for example) can explain differences in sensitivity to phthalate effects.

Epidemiological studies

Four studies have focused on abnormalities of the newborn and infant by looking for exposure during gestation and the first months of life.

For the first time in 2008, a study showed a link between the highest levels of metabolites (MEP, MBP, MEHP) and oxidized metabolites (MEHHP, MEOHP) in mothers at 29 weeks of gestation (on average) and a shorter anogenital distance (measured between the center of the anus and base of the penis) in male newborns. Concerning abnormalities of the genital system in young boys (hypospadias, cryptorchidism), very few studies with a sufficient number of cases have been conducted and it is therefore not possible to conclude on whether or not phthalates played a role in the occurrence of these problems.

In young girls, an effect on early puberty has been analyzed and the studies' findings are inconclusive.

In adult men, several cross-sectional studies find a link between the urine metabolite concentrations of phthalates and an alteration in the sperm parameters, including the sperm morphology and count as well as an increase in the DNA fragmentation of the male gamete. Two studies do not reveal any link between the urine phthalate concentrations and sperm parameters. One exposed/unexposed type study reveals a link between higher urine metabolite concentrations of phthalates and lower testosterone concentrations.

In women, few studies have evaluated the possible role of exposure to phthalates on reproductive health. Only the risk of endometriosis has been specifically assessed and evidence provided by these few studies of the possible existence of a link between phthalates and endometriosis is insufficient. The effects of phthalate exposure on ovulatory function and some hormone levels (estradiol, progesterone, LH, FSH) suggested in animal studies are not mentioned in women.

The evaluation of phthalate effects is complicated by the diversity of studies, populations studied, dosing method and detection limits, as well as the phenotypic parameters taken into account.

Studies in male animals

Benchmark studies used to determine the TDI for DEHP and DBP have been performed on three lines of rat (Sprague-Dawley, Wistar and Fisher). These were exposed during gestation and/or the neonatal period or over several generations. An impact on the testis and pathologies of the minor male sex organs such as the seminal vesicles, prostate and epididymis have been reported at various developmental

stages. Many studies report a fall in prostate weight, reduction in anogenital distance, increase in hypospadias or cryptorchidism, retention of breast areolas or nipples and a reduction in penis length for example. The effects are reported at DEHP and DBP doses of 150 and 500 mg/kg/d. The same effects can be obtained at lower doses (100 mg) when there are two phthalates present. Furthermore, it seems that some rat lines can be sensitive to doses of 10 to 100 mg/kg/d.

Alterations in sperm characteristics (sperm levels, morphology) and hormone levels (testosterone, LH, etc.) are observed after exposure to DEHP and DPB (doses over 100 mg/kg/d) in rats and rabbits. DBP reduces fetal testosterone function, but this effect disappears rapidly once gavage has ceased. Most of the studies on testosterone production during postnatal life show that, in adulthood, plasma testosterone levels of males treated in utero are comparable to those of unexposed animals. A fall in fertility is only reported for high doses (exceeding 500 mg/kg/d).

In male monkeys (marmoset, cynomolgus), these effects are found after exposure to both these phthalates (at the same doses) but not as evidently as in rats.

Target tissues and organs in males

The vast majority of studies on rats, performed by gavage during gestation, report effects on the three main cell types of the fetal testis: Leydig cells, Sertoli cells and germ cells. They are consistent in reporting abnormal Leydig cell aggregation and reduction in the testosterone and INSL3 production of these cells during fetal life, which inhibits masculinization or the development of androgen-dependent reproductive organs. This effect has not been observed in mice.

Regarding Sertoli cells, their number and proliferation may be inhibited temporarily during in utero exposure in the rat, without it being clear if this is a direct or indirect effect via inhibition of androgen synthesis.

Concerning fetal germ cells, the appearance of multinucleate cells in rats and mice has been observed after in utero exposure to DEHP and DPB. DEHP also increases the apoptosis of these cells. This effect is described in the rat and has also been found in organotypic cultures of human and murine fetal testis.

In the monkey (marmoset) and rat, delayed differentiation of fetal germ cells has been reported but no notable effect on the proliferation of Sertoli cells has been described in the monkey.

Studies in female animals

Few studies have focused on the effects of phthalates on the reproductive system of female animals.

In female rats, after in utero and neonatal exposure to DEHP, early or delayed puberty has been observed depending on the moment of administration (for doses of 15 or 150 mg/kg/d) and alterations in hormone levels (estradiol, progesterone). A reduction in fertility is reported with DBP after exposure from weaning and onwards (500 mg/kg/d).

In marmoset females exposed to DEHP just after weaning (3 months) and until sexual maturity (18 months), morphological analyses of the female genital tract reveal an increase in ovary and uterus weight at high doses as well as an increase in circulating estradiol levels. On the other hand, no significant change has been observed in histology in the uterus or ovaries, other than a slightly higher number of corpora lutea.

The increase in estradiol levels observed in the rat and marmoset could be the cause of the follicular atrophy observed in the ovary.

Target tissues and organs in the female

Data on female animals indicates that the ovary is a target organ for phthalates (DEHP, DBP). In vitro studies clearly show a reduction in estradiol production by ovarian follicular cells following exposure to DEHP and its main metabolite MEHP. Aromatase (the enzyme which converts testosterone into estradiol) could be a direct target for DEHP.

Other organs than the ovary are also potential targets, like the hypophysis or hypothalamus in the brain or the uterus and the mammary gland, as demonstrated in a few in vitro studies.

Studies in fish

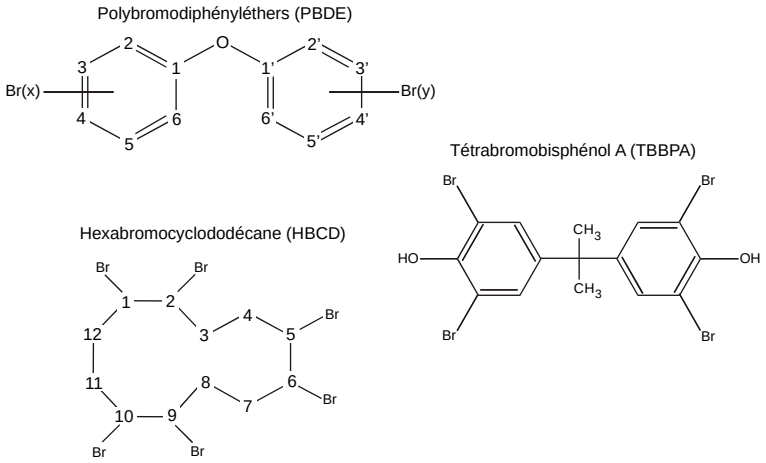
In the male zebrafish, DEHP exposure induces a reduction in the fertilization success of eggs laid by untreated females and spermatogenesis abnormalities (suggesting that meiotic progression might be disrupted). Other effects are commonly observed after BBP treatment: an alteration in the quality (motility and morphology) of sperm, a low quantity of ovotestis (gonad comprising both testicular and ovarian aspects), histological abnormalities of the male gonad and testicular differentiation abnormalities.

In the female zebrafish, the effects observed after DEHP treatment are more evident than those described in the male: sharp fall in the number of mature ovocytes, alteration of ovocyte growth and maturation, and more generally decreased capability to produce embryos.

Polybrominated compounds (flame retardants)

There are many types of flame retardants acting either chemically or physically. These are incorporated, in the products and materials concerned (fabrics, curtains, clothes, seats, plastics, foams, padding, resins, printed circuit boards, cables, televisions, computers, etc.), at levels generally ranging from 5 to 20%.

Chemical flame retardants belong to various families, the most commonly used of which are polybrominated compounds (BFRs) such as polybromodiphenylethers (PBDE), 1,2,5,6,9,10 hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA). They account for 30% of flame retardants in Europe.



Chemical structures of the main families of brominated flame retardants

From the viewpoint of their chemical structure, PBDEs are bicyclic aromatic ethers. They are classified according to the number and position of bromine atoms in the ring. To differentiate them, the usual nomenclature involves designating them by a number providing information about the degree of bromination (number of bromine atoms in the molecule) and the position of bromine atoms on the benzene rings.

Since August 2004, the European regulations have banned the two "technical" mixtures of PBDE used industrially (octa-BDE and penta-BDE) but still authorize deca-BDE. The latter mainly comprises BDE 209 or decabromodiphenylether, which can break down into less brominated congeners by physical degradation (particularly under the influence of UV rays).

TBBPA has a similar chemical structure to BPA, with two aromatic rings linked by a carbon bridge. When there are two hydroxylated groups, this compound becomes more polar than the other representatives of the family of brominated flame retardants, thereby setting it apart in terms of physicochemical properties. In particular, TBBPA is less bioaccumulable in fatty tissue and more subject to phase II metabolism reactions (conjugation) than PBDEs.

HBCD is in third place in the classification of the most commonly used brominated flame retardants. "Technical" HBCD is a mixture mainly comprising three diastereoisomers (identical compounds except as regards the spatial arrangement of their atoms). The γ isomer is found predominantly in biological samples taken from animals.

Polybrominated flame retardants are generally characterized by the physicochemical properties that make them lipophilic and bioaccumulable, in the same way as some other halogenated persistent organic pollutants (POPs) (dioxins, polychlorobisphenyls).

Although the production of some BFRs (particularly Penta-BDE and Octa-BDE mixtures) has ceased in some countries including Europe, their presence has increased in recent decades in the environment, in wildlife and in humans.

At present there is no TDI defined by the health agencies. Evaluations are under way. For information, the reference values issued by the American agency (USEPA) are 0.1 µg/kg bw/d for BDE 99 and 7 µg/kg bw/d for BDE 209.

Exposure

Because these pollutants are lipophilic, high-fat products (such as meat, fish and milk) make a major contribution to human exposure to brominated flame retardants. That said, the highly diverse eating habits from one continent to another explain a disparity observed in the main contributors to exposure.

Airborne (though dust ingestion) and direct (through contact with some manufactured products) exposure pathways are other ways in which humans are exposed to the most brominated congeners such as BDE 209, particularly young children.

In the general population, exposure levels to PBDE are estimated to be around 1 ng/kg of body weight/day.

Several congeners in some human biological fluids and tissues have been found. In serum or breast milk, levels observed are around a few ng/g of lipid.

Since the early 2000s, a downward trend in internal doselevels has been reported for the main PBDE type congeners, corresponding to a cessation in the production and use of the two industrial mixtures Penta- and Octa-BDE. However, this reduction does not seem to concern the other compounds that are still used, especially BDE 209, HBCD or TBBPA for which the available data is limited. According to the evaluation reports available, exposure levels to HBCD are from 2 to 10 ng/kg/day from fabric dust and 20 ng/kg/day via food.

For TBBPA, the exposure level is 80 ng/kg/day via the environment.

Studies performed on rats indicate that, after oral administration, BDE 209 is mainly eliminated in the feces whereas urinary elimination is insignificant. The half-life of BDE 209 is estimated to be 2 days in the rat and 14 days in humans. Overall, the half-lives of PBDEs vary from a few weeks (BDE 209) to a few years (BDE 47). For the different congeners, hydroxylated metabolites form and glucuronide and sulfate conjugates are eliminated in the urine.

Epidemiological studies

The only brominated compounds analyzed are PBDEs. The Finnish and Danish mother-child cohort study reports a link between cryptorchidism observed in newborns and 7 compounds of the PBDE family measured in breast milk gathered between 1 and 3 months. The authors also indicate an increase in luteinizing hormone (LH) levels. Another mother-child cohort in California found that some women took longer to conceive when they had higher blood PBDE levels. However, these studies are still too few in number to constitute sufficient proof of a role played

by PBDEs in male and female reproductive health. No study has considered exposure to other polybrominated flame retardants (HBCD and TBBPA).

Studies on animals

There are too few animal studies available, and most of these come from the same laboratory.

After exposure in utero to the compounds BDE 99 and 209 (500 and 1,500 mg/kg/day), male rats showed the following abnormalities: reduction in anogenital distance, alteration in sperm production and certain functional sperm parameters and reduction in testosterone levels.

Female rats showed a reduction in the number of ovarian follicles when exposed to the same compounds. The doses used (1 to 10 mg/kg sub-cutaneously) are much higher than the exposure estimated in humans however.

Target tissues and organs

In females, the ovary is a target organ. On a culture of isolated granulosa and thecal sow cells (or co-culture), exposed to a mixture of PBDE, a stable alteration after 48 h has been demonstrated in the testosterone and progesterone levels, which is likely to trigger early luteinization of preovulatory follicles. After chronic exposure of female rats to HBCD (140 mg/kg/day), following a histological examination of the ovaries, one study reports a reduction by one third of the primordial follicles in the ovarian reserve. A reduction in the number of primordial follicles is considered to be a biomarker of adverse effects on female reproduction as it is irreversible. But for all that, these female animals have a normal number of implantations during their first gestation. A study over a longer period of the animal's life would make it possible to determine whether the reduction in the follicular reserve causes premature ovarian failure.

In males, the target organ is the testis. In mice, exposure to BDE 209 (500 and 1,500 mg/kg/day) alters the mitochondrial membrane potential of epididymal sperm and the extent of the head's lateral movement, suggesting oxidative stress. In vitro studies with TBBPA on mouse Sertoli cells have shown cell death partly by apoptosis, implying a dependent CA^{2+} mitochondrial depolarization. These effects manifest at concentrations around 100 times higher than the highest levels of TBBPA measured in humans.

Perfluorinated alkylated substances

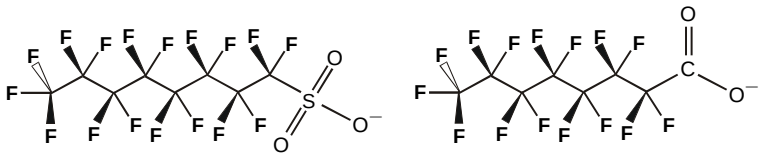
The term " perfluorinated alkylated substances " (PFAS) refers to a vast family of chemical molecules including oligomers and polymers. These are surfactants, neutral or anionic compounds presenting significant thermal, chemical and biological stability.

Perfluorinated alkylated substances used to be or still are used in a wide range of industrial applications, particularly for fabric stain-removal and waterproofing

treatments (clothing, materials, rugs or fitted carpets, etc.), fat-resistant coatings, paper and/or cardboard packaging authorized for food contact, non-stick coatings, fireproof foam, surfactants used in mining operations and oil wells, wax for wooden floors and some insecticide formulations. As a result, consumers in industrialized countries are currently in contact with such compounds on a daily basis through countless manufactured products. Like many other manmade chemical pollutants, perfluorinated alkylated substances can be released into the environment at every stage of their life cycle and then found once again in the food chain – and ultimately in living organisms.

A major sub-group of this family of emerging chemical pollutants for the past few years has been that of organic surfactants, like perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), which have a carbon chain with eight carbon atoms. Since both of these substances are the main end degradation products of numerous perfluorinated alkylated substances, they can be found in a generally dominant manner in environmental or biological matrices and are usually the only ones to be studied.

Because of their chemical structure comprising, on the one hand, a nonpolar polyfluorinated carbon chain and, on the other, a strongly polar group, PFCs are characterized by both hydrophobic and lipophobic properties, which means that they do not build up in the fatty tissue. The half-life of PFOS and PFOA is several years in humans, however. The former was recently included in the list of persistent organic pollutants of the Stockholm Convention (UNEP United Nations Environment Programme[10]). Since 2006, PFOS has been banned in some uses by European regulations.



Sulfonate de perfluorooctane Acide perfluorooctanoïque

Chemical structures of PFOS (perfluorooctane sulfonate) and PFOA (perfluorooctanoic acid)

In its scientific report published in 2008, EFSA's Contam panel set a tolerable daily intake value equal to 0.15 µg/kg/day for PFOS and to 1.5 µg/kg/day for PFOA.

Exposure

Diet appears to be the main exposure pathway to perfluorinated alkylated substances, particularly for adults. Other sources – particularly via direct contact with some rug or fitted carpet type coatings – nonetheless account for a not insignificant exposure pathway for young children. In general, the estimated exposure values to perfluorinated compounds vary from a few ng to a few dozen ng/kg/day. They appear to be below the tolerated limits for adults in the general

[10] http://chm.pops.int/Convention/POPsReviewCommittee/Publications/tabid/345/language/ fr-CH/Default.aspx

population but not far off the limits for particularly exposed sub-populations. For people categorized as high fish consumers, dietary PFOS intake via fish is estimated to be 0.2 µg/kg/day.

Several representatives of this class of compounds in some human biological fluids and tissues have been found. In serum, the levels observed are around a few ng to a few dozen ng/ml. PFOS and PFOA appear to be the main biomarkers of exposure to perfluorinated compounds.

A downward trend in internal dose levels in the general population has been observed in the United States since 2002, when production by one of the main producing companies was stopped. That said, because there is no data concerning other countries, this observation cannot be generalized.

Several studies have reported mother-fetus transfer via umbilical cord blood. However, the concentration levels reported in the umbilical cord blood are systematically lower than the levels observed in the mother's blood (by a factor of 1.5 to 3.5). Exposure of breastfed infants via breast milk has also been demonstrated, even if this mother-to-child transfer pathway appears to be more limited than for other classes of halogenated organic pollutants such as dioxins, PCBs or brominated flame retardants.

The estimated plasma half-lives for PFOS and PFOA are around a few hours in rodents, a few days in primates and a few years in humans. Of the bioaccumulable pollutants, they are less persistent than other more lipophilic substances such as dioxins or PCBs. Faster elimination in females has been demonstrated in animals, even though this difference is less significant in humans. Moreover, a higher inter- and intra-species variability can be observed in the pharmacokinetic parameters depending on compound, particularly according to the length of carbon chain (longer-chain compounds are more persistent).

Epidemiological studies

There are a very limited number of studies on the potential effects of PFOA and PFOS on the human reproductive function. One study carried out on a Danish birth cohort reports an association between plasma PFOS and PFOA levels and couple fertility (increase in the risk of involuntary childlessness). Another Danish study suggests a link between combined levels of PFOS and PFOA and a change in sperm morphology.

Studies on animals

Studies conducted on two generations indicate that, even at very high doses, PFOA does not alter male rat fertility. This concerns doses 30,000 times higher than the concentrations measured in humans. No harmful effects of PFOA or PFOS have been reported on rodent sperm production. The inter-species differences for half-lives and metabolism in particular need to be highlighted between humans and rodents.

In rats, two studies involving PFDoA (perfluorododecanoic acid) being administered by gavage indicate a fall in testosterone production (with a reduction in the

expression of genes coding for the testosterone biosynthesis enzymes) from the 0.2 mg/kg/d dose. Over 40 proteins (involved in oxidative stress and the mitochondrial respiratory chain) are modified in the testes of rats treated chronically.

Perfluorinated alkylated substances (PFOA and PFOS) do not seem to alter female fertility or ovarian morphology, but may alter ovarian steroidogenesis. In mice, delayed sexual maturity is observed in females treated (10 mg/kg/day) in utero: the age of vaginal opening and that of the first estrous cycle are delayed. An effect on the regularity of estrous cycles has been reported with PFOS (1 to 10 mg/kg/day) administered to adult female rats. These effects appear to be variable depending on the species (rat or mouse).

Target tissues and organs

In the adult rat testis, after treatment with PFDoA, reduction in testosterone production has been associated with an increase in apoptosis in different types of cells.

In mice, several studies demonstrate the effects of PFOA on the development or differentiation of the mammary gland (increase in the number of terminal end buds). This would seem to be related to the increased synthesis of ovarian progesterone under the effect of PFOA. Differences between mice lines have been reported.

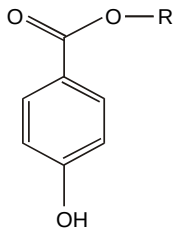
As a general rule, the effects of perfluorinated alkylated substances depend on their chemical structure and vary between species (rat, mouse) and lines.

In the adult male minnow, an increase in vitellogenin has been observed after treatment with PFOA. Signs of ovotestis have also been observed, which clearly suggests estrogenic activity. In females treated with PFOS, one study describes a reduction in gonad growth and alteration in the development of embryos laid by these females. Furthermore, PFOS induces histological abnormalities of the ovaries in the females treated.

Parabens

Parabens are 4-hydroxybenzoic acid esters that present a para-substituted benzene ring by an ester group with variably-sized alkyl chains. The most common structures are: methylparaben, ethylparaben, propylparaben, butylparaben and benzylparaben.

Because of their antibacterial and antifungal properties, parabens are very widely used as preservatives in cosmetics, drugs and food. They were first used as such back in 1920.



Chemical structure of parabens
R = alkyl chain (methyl, ethyl, propyl, butyl...)

The compounds of the paraben family that are authorized to be used as food additives are: methylparaben (E218) and its sodium salt (E219) and ethylparaben (E214) and its sodium salt (E215). Their use as food additives is governed by European Directive 95/2/EC of February 20, 1995.

The most commonly used parabens in cosmetics are methyl-, ethyl-, propyl-, butyl- and isobutylparaben. Directive 76/768/EEC governs the use of parabens in cosmetics and sets their use at 0.4% (in acid) for an ester and 0.8% (in acid) for ester mixtures. In drugs, propylparaben is the most commonly used.

The European health authorities (EFSA) have defined a tolerable daily intake as high as 10,000 µg/kg/day for methyl- and ethylparabens.

Exposure

Since they are used as preservatives in over 80% of cosmetics (shampoos, moisturizing creams, shaving foams, etc.), in countless pharmaceutical specialties and as a food additive, humans are exposed to parabens on a regular basis.

Exposure to methyl-, ethyl-, propyl- and butylparabens was assessed in a representative sample of the general population in the United States (participants aged 6 years and over) between 2005 and 2006. Methyl- and propylparaben were detected in over 90% of samples, and ethyl- and butylparaben in a little under 50%. The median concentration of methylparaben was 63.5 µg/l of urine and that of propylparaben – 8.7 µg/l. Teenage girls and adult women had significantly higher concentrations than teenage boys and adult men.

An estimate from the different possible sources of exposure indicates a rate of 1,300 µg/kg/day for the American population.

The confirmation of the ability of parabens to be absorbed systematically from topical applications has been demonstrated in humans: n-butylparaben is detected in the serum within 1 hour and in the urine with a peak at 8-12 hours, most of which is in the form of glucuronide conjugate.

Epidemiological studies

Regarding butylparaben, one study conducted on a hundred or so men, seeking medical help for infertility, has shown that its presence in the serum is significantly associated with alterations in sperm DNA. A dose-dependent relationship is observed with the increase in DNA fragmentation. However, there is not enough epidemiological data to confirm the impact of parabens on sperm quality.

In 2004, detection of the five most commonly used parabens (methyl-, ethyl-, n-propyl-, n-butyl- and isobutylparabens) in the fat of breast carcinomas triggered a scientific and societal debate on the possible effect of some compounds from the

paraben family used in local skin application under the armpits (particularly because of their presence in deodorants) on the risk of breast cancer occurring. This study has been criticized because it neither contained many samples, nor any controls.

What's more, two epidemiological studies (2002, 2003) do not provide any evidence concerning the possible impact of parabens present in deodorants or antiperspirants on the occurrence of breast cancer. The first, a case-control study, did not report any increase in risk associated with the use of deodorants/antiperspirants. The second, in women with breast cancer, only observed a link between the age at which this cancer occurred and the early use of deodorants/antiperspirants (associated with shaving). At present, none of the findings available enable a response to be given to this question.

Studies on animals

Concerning methylparaben, one study conducted by an industrial consortium has recently concerned the absence of effect on male reproductive organs after oral administration in young rats (1,000 mg/kg/day).

For propylparaben, an effect on spermatogenesis with no alteration in the weight of the male reproductive organs has been reported: reduction in the testicular and epididymal quantity of sperm (around 50% of controls at a dose of 1,000 mg/kg/day); reduction in the daily production of sperm in all groups (around 70% of controls); dose-dependent reduction in the serum testosterone concentration. This study has been criticized in several health agency evaluations because of the few animals used, lack of details provided and major variations in animal weights and hormone dosages... It should also be noted that the study duration does not cover a complete spermatogenesis cycle (52 days in rats).

Regarding butylparaben, a recent study on rats, performed under satisfactory experimental conditions has demonstrated the absence of effect on male reproductive organs. In mice, no effect of butylparaben has been described on the weight of the prostate, seminal vesicles and preputial glands. However, the weight of the epididymis did appear to increase slightly at the highest dose (1,000 mg/kg/day). A dose-dependent reduction in spermatids in the seminiferous tubules has been observed, with no change in the number of spermatogonia or spermatocytes. Serum testosterone is significantly reduced at the 1,000 mg/kg/day dose.

Some studies have analyzed the effect of parabens on the female reproductive parameters. One recent study indicates that a high intake of methyl- and isopropylparaben (1,000 mg/kg/day) significantly delays vaginal opening (sign of puberty in female rats) and reduces the estrous cycle length. This high intake of the two compounds is responsible for a reduction in ovarian weight, lack of corpus luteum and increase in the number of cystic follicles. The histological analysis finds abnormalities in the uterus, namely a hypertrophy of the myometrium for the highest dose of propyl- and isopropylparaben (1,000 mg/kg/day) and for all doses of butyl- and isobutylparaben (62.5, 250, 1,000 mg/kg/day). Estradiol levels are significantly reduced in animals treated with methyl-, ethyl-, propyl-, isopropyl- and isobutylparabens. The other studies mainly show an increase in uterus weight.

Review and limits of studies on humans and animals

There are still too few epidemiological studies to date that have looked for links between the five chemical families analyzed in this expert report (bisphenol A, phthalates, polybrominated compounds, perfluorinated compounds and parabens) and abnormalities of the male and female reproductive system, reproductive disorders and human fertility for any conclusion to be drawn as to the effects of exposure to these relatively recent compounds. Until new epidemiological studies are conducted, animal testing remains an invaluable source of data.

Studies on animals, particularly rats and mice (sometimes non-human primates), performed in different contexts have reported effects on the male and female reproductive system, on sperm production and quality, and sometimes on fertility. In those studies having demonstrated effects, the exposure period in utero and until weaning appears to be the most critical. Some effects are associated with very precise exposure periods (in terms of days), at the end of gestation for example (phthalates). Alterations in the levels of such hormones as testosterone, which plays a vital role in masculinization of the genital tract, can return to normal in adulthood. However, other early effects can lead to pathological consequences a long time after exposure.

One of the first parameters complicating the analysis of chemical substances that are potentially toxic for reproduction is their specific action depending on the developmental stage. For example, some substances affect the fetal gonad more or specifically in comparison with the adult gonad. It is even possible to observe differences in effects a few days apart during the development of the fetal gonad.

Cellular effects of the different chemical substances

A process disruption at cellular level may cause fertility problems or explain the occurrence of precancerous lesions. Chemical substances can act directly on certain types of cells via their differentiation, proliferation, interaction and survival, without altering hormones.

Apoptosis or programmed cell death

In the case of gonad development, several chemical substances are suspected to increase apoptosis within the germ line, which leads to a reduction in the number of these cells and therefore a person's potential to reproduce. This process, if not offset by the proliferation of surviving cells, may bring about a reduction in the number of gametes and therefore hypofertility. Bisphenol A and some phthalates can increase male germ cell apoptosis during development.

On the other hand, a reduction in apoptosis can have pathological consequences. As such, the appearance or development of "precancerous" lesions is sometimes attributed to a lack of apoptosis. This hypothesis has been put forward in the case of female mice exposed to bisphenol A at the end of fetal life or during puberty, contributing to the development of lesions observed later on in the mammary glands.

Furthermore, the effect on apoptosis can vary depending on age or developmental stage. In mice, MEHP (phthalate metabolite) increases male fetal germ cell apoptosis at 13.5 and 18.5 days postconception but has very little effect, at the same dose, at 15.5 days postconception. Such highly specific windows of action are commonly described for male fetal steroidogenesis and gametogenesis (leading to masculinization).

Cell proliferation

A disturbance in cell proliferation can also bring about fertility problems or be suspected in the occurrence of cancers. Bisphenol A stimulates the proliferation of human seminomas. Phthalates (such as DBP) are able to reduce the proliferation of Sertoli cells in the rat, and it is known that an adult's sperm reserve depends on this.

Cellular differentiation

In males, in the germ line, cells undergoing mitosis (division of fetal or adult germ stem cells) express numerous stem cell markers. At the time these cells differentiate, they lose these pluripotency markers. Obstructed differentiation of fetal germ cells is correlated with the occurrence of testicular tumors. It has been suggested that phthalates (DBP) obstruct or delay the differentiation of male fetal germ cells in rats. However, no testicular cancers have been found in rats exposed to phthalates, perhaps because of the rarity of this type of cancer in rodents outside of certain highly specific genetic bases.

In females, the accelerated recruitment of primordial follicles following bisphenol A exposure and their premature engagement towards subsequent differentiation stages will reduce the gamete stock and lead to early menopause. Lastly, alterations in the differentiation of hypothalamic neurons have been demonstrated in male and female animals, with long-term consequences on the secretion of gonadotropic hormones at puberty and in adulthood.

Mechanisms of action: modification in the hormonal function (endocrine disruption)

Most of the chemical substances discussed in this expert report are designated as being endocrine disruptors. There are several definitions for endocrine disruptors. According to the European Commission, an endocrine disruptor is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function (European workshop on the impact of endocrine disruptors on human health and wildlife, Weybridge, UK, 1996). According to the US Environmental Protection Agency (EPA), an endocrine disruptor is an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes. This definition does not mention any adverse effect on the body or its progeny.

The first definition poses methodological difficulties as it requires knowing both the impact of a substance at fundamental level and checking that this substance has an effect on the occurrence of a disorder via this mechanism. Yet, of the many potential mechanisms of action of endocrine disruptors, only a few have been associated with "phenotypes" with regard to the reproductive system.

The action of chemical substances on reproduction may be direct or indirect depending on whether they act on gonads or on the hypothalamo-hypophyseal axis which controls gonadic hormonal secretions. They can act at these two levels as well as on other organs such as the thyroid, which will have an effect on testicular function for example.

The majority of studies focus on the binding of substances to the nuclear receptors of steroid hormones (ER and AR), thereby disrupting the binding of natural ligands – hormones. The effects can be estrogenic or antiandrogenic. Estrogen-type substances can cause structural or functional abnormalities in males and females during development while androgen-type substances mainly affect females, and antiandrogens – males.

Major research efforts have been undertaken to characterize substances that alter the signaling of "canonical" nuclear receptors of estrogens and androgens.

Potential hormonal activity of the 5 families studied

Hormonal activity	Agonistic	Antagonistic
Estrogenic	BPA, Phthalates, Parabens	
Androgenic		Phthalates, BPA, Parabens
Thyroidal		TBBPA

However, other methods of action are possible via less conventional hormonal receptors (membrane forms of these nuclear receptors, GPR30), via other orphan nuclear receptors such as ERR, PXR or CAR responsible for enzyme system induction to eliminate exogenous substances, or via receptors that control activation of peroxisome proliferation (PPAR).

Depending on dose, chemical substances can have different affinities for receptors, which can explain the inverted U-shaped activity or binding curves and can act via different mechanisms of action.

Nuclear receptors play a central role in regulating countless networks of genes involved in cell differentiation and proliferation, development, homeostasis and metabolism.

That said, in many cases, an alteration in the weight or structure of reproductive organs, in cell proliferation or in the hormonal secretion of a tissue, is reported without light having been shed on the mechanism of action involved. This is partly due to the fact that basic knowledge is still incomplete, including about the functions of nuclear receptors.

The effect on the signaling pathways of thyroid hormones, which are important regarding testis size and sperm production (proliferation of Sertoli cells), or retinoids,

which play a key part in determining the sex of germ cells, has been studied very little.

What's more, a substance may act on several signaling pathways, such as steroid signaling and retinoid signaling by binding to the same receptor (SHP, small/short heterodimer partner).

Bisphenol A

Bisphenol A is a weak estrogen agonist that can bind to estrogen nuclear receptors α and β (ERα and β). The affinity of bisphenol A for ERα and β is several thousand times weaker than that of estradiol. Some in vitro studies have shown that bisphenol A has antiandrogenic effects and can bind to the androgen receptor (AR) but evidence of antiandrogenic effects in vivo in animals is scarce. More recently, bisphenol A binding to other nuclear or membrane receptors has been described.

Nuclear receptors involved for the different substances studied

	BPA	Phthalates (DEHP, DBP, etc.)	Flame retardant (PBDE, TBBPA, HBCD)	Perfluorinated (PFOA PFOS)	Parabens
ER	(+)	(+)	(+) (-)		(+)
AR	(-)	(-)	(+) (-)		(-)
PPAR α or γ		(+)	(+) for TBBPA	(+)	
PXR	(+)	(+)	(+) (-)		
ERRγ	(+)				
TR			(+) (-)		
CAR		(+)		(+)	

(+): agonist; (-) : antagonist

Although the binding affinities are not very strong (with the exception of GPR30 if interaction is confirmed), synergistic actions between nuclear receptors (ERα, ERβ and AR) may explain low-dose effects. Moreover, the fact that bisphenol A binds to membrane forms of estrogen receptors suggests that it may also interact with membrane forms of other nuclear receptors such as AR, TRα or β.

A hydroxylated aromatic group (phenol ring) and hydrophobic skeleton, vital characteristics for estrogenic activity, are found in bisphenols. Furthermore, the nature of substituents linked to the carbon bridge is important for determining estrogenic activity. Chlorine derivatives that can form when thermal paper is recycled (by bleaching with sodium hypochlorite) have been described in some studies as being 28 times more estrogenic than bisphenol A.

Phthalates

Endocrine disruption of phthalates seems primarily to be due to their activity of PPAR receptor agonists (PPARα and PPARγ). Through this binding to PPAR receptors, the major effect of phthalates may be to inhibit aromatase expression. This antiestrogenic effect can be partially offset by activation of estrogen receptors and, on

the other hand, facilitated by inhibition of the androgen receptor. The alpha and beta estrogen receptors, androgen receptor, xenobiotic receptors CAR (constitutive androstane receptor) and PXR (pregnane X receptor) have been identified as possible mediators of these molecules' effects. Note that the diverse phthalates do not have the same interaction profile with the different nuclear receptors. As such, MEHP is mainly active on PPARγ and does not interact with ER receptors. On the other hand, BBP and DBP are active on ER and interact very little with PPARγ. Studies on mice models that are deficient for one or more of these receptors would shed more light on the mechanism of action of different phthalates (MEHP versus BBP for example).

Some phthalates present a moderated or even low binding affinity for the AR receptor in vivo, compared with steroids. Two anchoring points are required for there to be strong interaction (hydrogen type binding) with the AR receptor, and therefore high antiandrogenic activity. When these chemical characteristics are not present, only hydrophobic interactions allow interaction with the receptor. For phthalates, the aromatic ring can be involved in this type of interaction and a second benzene ring at a distance of three carbon atoms from the primary ring, such as in BBP, may strengthen this interaction.

Polybrominated flame retardants

The targets of flame retardants (PBDE, TBBPA, HBCD) on nuclear receptors have not been completely identified. The ER, AR and PXR receptors seem to be involved for PBDEs. The findings of several in vitro screening studies suggest that estrogenic activity is associated with low PBDE bromination, while antiestrogenic activity is determined by a high degree of bromination. The molecules substituted to positions [2,2′,6] or [2,2′,4] have an agonistic-ER activity. Likewise, the QSAR (Quantitative Structure-Activity Relationship) model links antagonistic-AR activity with a low degree of bromination and with ortho-substitutions (positions 2, 2′, 6 and 6′) or ortho- and para-substitutions (positions 4 and 4′). The TR and PPAR receptors are targets of TBBPA.

Perfluorinated alkylated substances In humans, the main targets of perfluorinated alkylated substances seem to be PPARs and, of these, PPARα and PPARγ. Studies have shown that the receptor PPARα is the main target of PFOA and PFOS, even though the PPARγ receptor is also activated to a lesser extent.

However, tests conducted on PPARα deficient mice indicate that part of the genes (5-10%) whose expression is adjusted by perfluorinated alkylated substances would seem to be controlled by the CAR nuclear receptor. One recent study has shown a tumor proliferative effect of PFOA which would also be independent of PPARα.

Parabens

As early as 1998, studies were also showing the ability of parabens to bind with the estrogen receptor (ER). That said, their binding affinity for the estrogen receptor is 10,000, 30,000, 150,000 and 2,500,000 times weaker for butyl-, propyl-, ethyl- and methylparaben respectively than the natural ligand, 17β-estradiol. In tests in vitro, intensity of the estrogenic activity measured increases with the length of the chain

(methyl<ethyl<propyl<butylparaben). Given their small size, two parabens (from methylparaben to n-butylparaben) may enter the receptor pocket and lead to synergistic effects. Very low antiandrogenic activity has also been observed in vitro with methyl-, propyl- and butylparaben.

Other mechanisms of action

A few years ago the idea first emerged that the effects of chemical compounds on reproductive organs and the reproductive function could occur without having to involve binding to a hormonal –whether nuclear or membrane - receptor.

It was also suggested that chemical compounds can compete for binding to transport proteins (SHBG, sex hormone-binding globulin) or alter the intracellular metabolism of hormones (aromatase), adjust the expression of co-activators of nuclear receptors, deteriorate these or modify gene expression through epigenetic mechanisms. Several studies have described alterations in epigenetic mechanisms involving DNA methylation, which might demonstrate transmission to future generations of some phenotypes.

Hypotheses have also been formulated about alteration of the cytoskeleton of some cell types and about a direct action targeting germ cells, without involving steroid disruption. It would be worth building on and delving deeper into these findings.

New research challenges

Methods for studying chemical substances that have a potential impact on reproduction can be intended for screening, to characterize the risk, and/or for studying the mechanisms of action.

Screening methods include in vivo and in vitro testing. Regulatory study methods have been defined and correspond to OECD (Organization for Economic Co-operation and Development) guidelines. There are six OECD methods (plus a seventh currently undergoing validation) as regards the impact of chemicals on reproduction. In vitro methods do not shed light on how chemical substances are metabolized and stored in the body, while in vivo studies factor in all of the interactions.

In screening, the study methods can be organized into two complementary levels. At the first level, simple and quick tests can be performed to identify those chemical molecules that have an endocrine disruption potential. This is, for example, the binding of steroid hormone receptors. The advantage of in vitro tests is that scientists can work on a very high number of samples and comply with the three R's rule (set by the European legislation on animal protection: reduce/replace/refine): reduce the number of animals; replace animals where possible; refine the method used.

At the second level, more detailed tests such as organotypic cultures and in vivo tests – which are less suitable for high-throughput screening – are more forthcoming about the complex effects bringing into play interactions within organs or the whole

body. At present, these are the only tests that can determine the link with a possible impact on fertility or pathological change.

To be able to select tests for predicting possible harmful effects on the reproductive function, knowledge must be improved of the fundamental mechanisms in play (there is still a long way to go in identifying all of the signaling pathways involved in the key stages of reproduction in mammals) and more understanding gained of the mechanisms of action of substances toxic for reproduction in situ in their target tissue(s).

It is important to develop cell lines corresponding to diverse reproductive tissues so as to be able to study these mechanisms of action, but to understand the link with fertility it is necessary to have access to models that are closer to physiology such as co-cultures, organotypic culture or in vivo testing.

In addition to in vitro and in vivo studies, in silico methods, or "expert systems", have emerged more recently which make use of IT tools. These methods, such as the QSAR ones, provide information about the nature of chemical interactions between the substances and their receptors, and are invaluable for understanding these very mechanisms. Subsequently, such studies can be used to set up new tests or when selecting which molecules to test, as well as fostering the search for safer substitutes (meeting the requirements of the European Regulation REACH).

It is often necessary to avail of a whole host of arguments for strengthening the relationship between a chemical substance's biological properties and its role in pathology. Moreover, knowledge about the method of action would tell us whether observations made in animals can be transposed to humans.

When transferring studies using in vitro models or laboratory animals to humans, the genetic disparity of the human population must be taken on board. This is because diverse polymorphisms in key players in the reproductive function are now known. Polymorphisms have also been described for androgen receptors and estrogen receptors (AR and ER) as well as for SF1 (steroidogenic factor 1). For several chemical substances, particularly phthalates, very different effects have been reported from one species to another, and sometimes even between two different animal lines of the same species.

Few studies (in vivo and in vitro alike) have addressed the crucial problem of effects of complex mixtures. Mixture effects (associated with a reality of multi-exposure), low-dose effects behind chronic exposure, non-conventional dose-effect relationships or the existence of multiple biological targets for the same substance all illustrate how complex the study of chemical substances is – particularly endocrine disruptors and the evaluation of potential risks.

As a result, methodological approaches based on the measurement or monitoring of a single parameter – whether this be a given chemical (characterization of exposure, metabolic and/or pharmacokinetic studies) or a clinical "end-point" (characterization of the risk, pharmacodynamic studies) – are usually unsuitable in this case.

Developments in toxicology towards more integrative approaches, tools and concepts are emerging. In this context, the use of comprehensive "omics" type profiling technologies probably represents a major development pathway for

toxicology in the years to come. Although the first two descriptive levels of biological systems – transcriptomics and proteomics – are also fairly widely used, the third (of the metabolome) is still recent. Metabolomic phenotyping approaches focus more specifically on small molecules (so-called metabolites) found in biological systems after the complex phenomena of transcription and translation have taken place. One objective of metabolomics can be then to identify biomarkers of exposure to different substances in such biological fluids as blood or urine, and/or biomarker of effects associated to a considered health trouble/pathology. Accordingly, one ultimate ambition of this biomarker discovery would be to determine whether one individual has been exposed at some point in his or her life and to predict the consequences of this exposure on his or her health, for instance his or her fertility.

Measuring these biomarkers as part of epidemiological studies provides significant data about the exposure levels of populations to environmental pollutants. Prospective cohort type approaches, which draw from large biobanks, have the advantage of being able to study different health parameters simultaneously and to evaluate exposure to chemical substances in a prospective manner. In the same way, it is important to highlight the merits of mother-child cohorts through which the child's health and sensitivity to exposure suffered during intrauterine life can be monitored over the medium- and long-term.

Approaches by complementary disciplines and interaction between epidemiologists, physiologists, toxicologists and reproductive biologists should paint a more integrated picture of the effects of substances and enable a response to be provided to society's reproductive health challenges through a series of methodologies:

• using conventional approaches to study the absorption, distribution and metabolism of toxins;

• using cell biological models similar to human physiological states (organotypic) and considering individual intervariability (iPS cells);

• using relevant animal models (mutant transgenic mice for a receptor) to understand the mechanisms of action on target tissues; or of non-rodent mammals or humanized transgenic animals that are more representative of the human situation;

• exploring the disruption of natural hormones' biosynthesis pathways to identify the different levels of modification with quantification tools;

• applying structural biology to understand the interactions of substances with their cellular protein targets;

• using high-throughput, genomic, proteomic and metabolomic appoaches allowing for a large-scale analysis of the potential molecular effects and for multiple exposure biomarkers to be sought;

• applying biochemistry and molecular biology tools to biobanks put together during epidemiological cohorts;

• applying systems biology that can integrate this information in a comprehensive mathematical model, to predict long-term effects;

• considering multi-exposure and using sensitive and easy-to-get indicators, such as sperm or the time needed to have a baby.

Appendix

Table: Summary of the main data

	BPA	Phthalates	Polybrominated compounds	Perfluorinated alkylated substances (PFOS, PFOA)	Parabens
Uses (a few examples)	Polycarbonate and epoxy resins Food packaging, dental composites, etc.	Plasticizers, construction, paints, cosmetics, food packaging, medical equipment, etc.	Flame retardants (fireproofing of plastics and fabrics, electronic components and electrical equipment, etc.)	Non-stick and stainproof applications (fabrics, cooking materials, etc.)	Preservatives (cosmetics, food additives, drugs)
Main exposure pathway	Food	Food; Percutaneous contact (children)	Food (remanent compounds); Direct contact (children)	Food (remanent compounds); Direct contact (children)	Percutaneous contact; Food
TDI (µg/kg/d) EFSA	50	DEHP: 50 DBP: 10 BBP: 500	Under way	PFOS: 0.15 PFOA: 1.5	0-10,000 (ethyl +methyl)
Average exposure (µg/kg/d)	Adults: 0.1 Children: 1	DEHP Adults: 2 Children: <6 months: 10 Children: >6 months: 20	PBDE: 0.001	0.005	1,300 (estimation)
Half-life	4-8 h (adult men)	DEHP: 18 h	A few weeks (BDE 209) to a few years (BDE 47)	A few years	A few hours
Effects/animals Males	Testicular hypotrophy, prostate hypertrophy, shorter anogenital distance, sperm abnormalities, abnormal hormone levels	Shorter anogenital distance, hypospadias, cryptorchidism, breast areola retention, sperm abnormalities, disrupted hormone levels	Shorter anogenital distance, sperm abnormalities, disrupted hormone levels	PFDoA: reduction in testosterone levels	Propylparaben: alterations in sperm parameters (discussed) Butylparaben: reduction in testosterone (discussed)

Effects/animals Females	Uterus, vagina, ovary and endometrium abnormalities; early puberty, abnormal hormone levels (cyclicity, ovarian function), behavioral disorders	Puberty (early or delayed), disrupted hormone levels (irregular cycles)	Reduction in ovarian follicles, abnormal hormone levels	PFOS/PFOA: disrupted hormone levels, delayed sexual maturity, effects on estrous cycles	Methyl isopropylparaben: effect on puberty Butyl, isobutyl: hypertrophy of the myometrium
Main target organs in males	Prostate (development), testis	Fetal testis	Testis	Testis	-
Main target organs in females	Hypothalamus, hypophysis, ovary, uterus, mammary tissue	Ovary, uterus	Ovary, uterus	Ovary, mammary tissue	Ovary, uterus
Critical periods in animals	In utero, neonatal period	In utero, neonatal period	In utero, neonatal period	In utero, neonatal period	Prepuberty and adulthood
Effects on the reproductive function in men	Possible effect on sexual function, sperm characteristics and hormone levels in adult men	Possible effect on anogenital distance, hypospadias, cryptorchidism (exposure in utero) Possible effect on sperm characteristics and hormone levels (adult exposure)	PBDE: possible effect on cryptorchidism and disrupted hormone levels (exposure in utero)	Possible effect on sperm morphology (adult exposure)	No effect demonstrated
Effects on the reproductive function in women	No studies of sufficient quality	Possible effect on early puberty (exposure in childhood)	Possible effect on increase in the time it takes to conceive (adult exposure)	Possible effect on increase in involuntary childlessness	No effect demonstrated
Disrupted hormonal pathway	Alteration in estrogen sensitivity	Antiandrogenic effect, estrogenic effect	Alteration in estrogen sensitivity and thyroid hormones	Alteration in estrogen sensitivity	Alteration in estrogen sensitivity
Key points	Transgenerational effects, low-dose effects, non-monotonic effect, binding to numerous receptors, effects revealed in the long-term	Possible additive effects, few studies on females, inter-species variability depending on compound and window of exposure	Not enough studies on authorized PBDE mixtures, on HBCD TBBPA; binding to numerous receptors	Not enough studies at compatible doses with exposure	Very few studies