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I. Context

Laboratoire Watchfrog was requested to review the estrogenic potential of the Hexane and its toxic metabolite, 2,5-hexanedione.

In order to achieve this goal, a bibliographic research was conducted and the following report synthetize the key information's from the scientific articles below.

II. Bibliographic summary

a. References

Abolaji, A.O. *et al.* (2015) Exposure to 2,5-hexanedione is accompanied by ovarian and uterine oxidative stress and disruption of endocrine balance in rats. *Drug Chem Toxicol*, **38**, 400–407.

Adedara, I.A. *et al.* (2014) Impairment of hepatic and renal functions by 2,5-hexanedione is accompanied by oxidative stress in rats. *J Toxicol*, **2014**, 239240.

Adedara, I.A. *et al.* (2016) Redox status of the testes and sperm of rats following exposure to 2,5-hexanedione. *Redox Rep*, **21**, 239–247.

Bilgi, A. *et al.* (2019) The apoptotic effects of bisphenol A exposure on the rat ovary: an experimental study. *Environ Sci Pollut Res Int*, **26**, 10198–10203.

Hassani, S. *et al.* (2014) Menstrual disturbances and hormonal changes in women workers exposed to a mixture of organic solvents in a pharmaceutical company. *Med J Islam Repub Iran*, **28**, 156.

Howe, G.R. and Morello, C.J. (1985) Effects of an anabolic steroid on reproduction in female rats. *Steroids*, **45**, 497–501.

Huang, H. *et al.* (2011) The impact of n-hexane on the secretion of mouse estrogen and progesterone. *Advances in Bioscience and Biotechnology*, **2**, 429–433.

Iba, M.M. *et al.* (2000) Functional Cyp2e1 is required for substantial in vivo formation of 2,5-hexanedione from n-hexane in the mouse. *Arch Toxicol*, **74**, 582–586.

Jernigan, J.D. and Harbison, R.D. (1982) Role of biotransformation in the potentiation of halocarbon hepatotoxicity by 2,5-hexanedione. *J Toxicol Environ Health*, **9**, 761–781.

Li, H. *et al.* (2015) Gestational N-hexane inhalation alters the expression of genes related to ovarian hormone production and DNA methylation states in adult female F1 rat offspring. *Toxicol Lett*, **239**, 141–151.

Liu, J. *et al.* (2012) The effect of n-hexane on the gonad toxicity of female mice. *Biomed Environ Sci*, **25**, 189–196.

Nieschlag, E. and Vorona, E. (2015) MECHANISMS IN ENDOCRINOLOGY: Medical consequences of doping with anabolic androgenic steroids: effects on reproductive functions. *Eur J Endocrinol*, **173**, R47-58.

Nylén, P. *et al.* (1989) Testicular atrophy and loss of nerve growth factor-immunoreactive germ cell line in rats exposed to n-hexane and a protective effect of simultaneous exposure to toluene or xylene. *Arch Toxicol*, **63**, 296–307.

Qi, B. *et al.* (2019) Proapoptotic effects of 2,5-hexanedione on pheochromocytoma cells via oxidative injury. *Mol Med Rep*, **20**, 3249–3255.

Ruiz-García, L. *et al.* (2020) Possible role of n-hexane as an endocrine disruptor in occupationally exposed women at reproductive age. *Toxicol Lett*, **330**, 73–79.

Sallmén, M. *et al.* (2008) Reduced fertility among shoe manufacturing workers. *Occup Environ Med*, **65**, 518–524.

Sallmén, M. *et al.* (1995) Reduced fertility among women exposed to organic solvents. *Am J Ind Med*, **27**, 699–713.

Siracusa, G. *et al.* (1992) Effects of 2,5-hexanedione on the ovary and fertility. An experimental study in mice. *Toxicology*, **75**, 39–50.

Wolf, C. *et al.* (1999) Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol Ind Health*, **15**, 94–118.

Zaroogian, G. *et al.* (2001) Effect of 17beta-estradiol, o,p'-DDT, octylphenol and p,p'-DDE on gonadal development and liver and kidney pathology in juvenile male summer flounder (Paralichthys dentatus). *Aquat Toxicol*, **54**, 101–112.

b. Conclusions

It has been well demonstrated that n-hexane and its toxic metabolite 2,5-HD are reprotoxic in both male and female humans, laboratory animals and *in vitro* in explant tissue culture. A number of studies have also concluded that exposure to these molecules causes alterations in circulating sex steroid levels. At present the cause-and-effect relationship between the change in hormone levels and observed atrophy and degeneration of the gonads has not been deduced. Deduction of the relationship between these events will allow these molecules to be classified as either reprotoxins or endocrine disruptors.

Given the information available on the modes of action of hexane and 2,5-HD, it is not possible to discern whether the molecules are endocrine disruptors or reprotoxic. However, they are systematically suspected of being endocrine disruptors, until proven otherwise.

In order to rule out all estrogenic activities on this type of molecule or substitute molecules, it seems necessary to assess their estrogenic activity *via* as many available modes of action as possible.

c. Definition of hexane

Hexane is a six-carbon organic molecule with the molecular formula C_6H_{14} it exists as a number of isoforms as shown below in table 1.

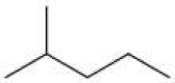
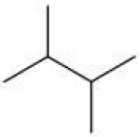
Common name	IUPAC name	Text formula	Skeletal formula
normal hexane *n*-hexane	hexane	$CH_3(CH_2)_4CH_3$	
isohexane	2-methylpentane	$(CH_3)_2CH(CH_2)_2CH_3$	
	3-methylpentane	$CH_3CH_2CH(CH_3)CH_2CH_3$	
	2,3-dimethylbutane	$(CH_3)_2CHCH(CH_3)_2$	
neohexane	2,2-dimethylbutane	$(CH_3)_3CCH_2CH_3$	

Table 1: Hexane molecular formula and isoforms.

d. Definition of an endocrine disruptor

In 2017 the European Union (EU) ratified the following definition of an endocrine disruptor:

"An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (EU Regulations 2017/2100 and 2018/605).

This definition is very similar to definitions which had previously been validated by the World Health Organisation (WHO) and the endocrine society.

According to the EU definition, a chemical is considered as an endocrine disruptor if it meets two criteria. It must alter the function an element of an endocrine system and it must alter it to a degree that causes adverse health effects. The requirement that the endocrine disruption results in an adverse health effect prevents chemicals inducing minor alterations in hormonal signalling, which are within a normal range that can be compensated to maintain homeostasis, from being considered as endocrine disruptors. A concrete example of a this would be consuming a tea spoon of sugar, which would alter levels of the endocrine hormone insulin within a normal range. This would not however result in adverse health effects and therefore would not be considered as an endocrine disruptor. The EU definition of an endocrine disruptor also requires a plausible mechanistic link to be determined between the alteration of one or more endocrine systems and the observed adverse health effects.

e. Is sufficient evidence available to classify n-hexane as an endocrine disruptor

While much attention has been paid to the neuropathic effects of both n-hexane and its toxic metabolite 2,5-hexanedione (2,5-HD), a number of studies since the 1970's have focussed on the reprotoxic effects of these molecules. It is widely accepted that the effects of n-hexane are mediated through *in vivo* metabolism to 2,5-HD (Jernigan and Harbison, 1982). The generation of 2,5-HD *in vivo* is via cytochrome P450 2E1 (Iba *et al.*, 2000, 1).

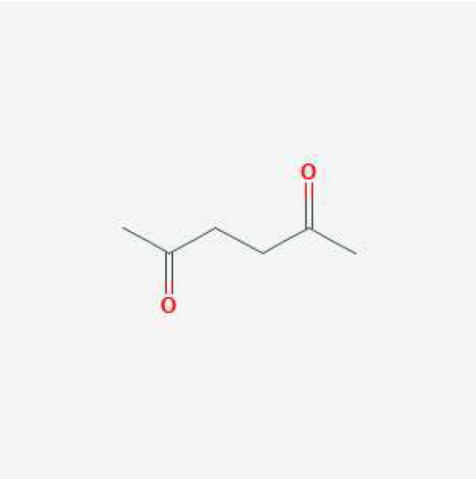


Figure 1: Skeletal formula of 2,5-hexanedione (2,5-HD).

Concerning the reprotoxic effects of n-hexane and 2,5-HD, the literature is very clear that metabolic generation of 2,5-HD can cause reprotoxic effects in both males and females. These effects would clearly be considered as adverse health effects in line with the definition of an endocrine disruptor.

The toxic metabolite 2,5-HD can cross the placental barrier and in some cases, at least in rats, the effects of this n-hexane inhalation have been shown to be transgenerational and to cause health effects in the following generation after gestational exposure (Li *et al.*, 2015). Reported adverse health effects include sever testicular atrophy and permanent loss of the germ cell line resulting in loss of reproductive capacity in male rats and reduced numbers of growing oocytes and reduced fertility in female mice (Siracusa *et al.*, 1992; Nylén *et al.*, 1989). The reduced fertility in female mice appears to be due to them producing fewer eggs and a reduction in ovarian follicles, with increased levels of apoptosis in the ovaries (Liu *et al.*, 2012).

In addition to the observed effects in animal studies, epidemiological studies in female workers occupationally exposed to n-hexane have identified hormonal changes, menstrual disturbances and reduced fecundity (Sallmén *et al.*, 1995, 2008; Hassani *et al.*, 2014). A study of Mexican women working in a shoe factory also identified a correlation between the longer time to pregnancy and longer menstrual cycles in exposed women (Ruiz-García *et al.*, 2020). The toxic metabolite of n-hexane, 2,5-HD, was detected in the blood of the exposed women and levels correlated with reduced levels of follicle stimulating hormone (FSH).

Concerning the second requirement of the definition of an endocrine disruptor, there is overall consensus that elements of the sex steroid axes are disrupted. For example, mice exposed *in vivo* to n-hexane had significantly lower serum levels of progesterone (Huang *et al.*, 2011; Liu *et al.*, 2012). The study performed by Huang *et al.* also involved *in vitro* 2,5-HD exposure of ovarian tissue taken from mice at different stages of the estrous cycle. As with the *in vivo* data, significantly lower levels of progesterone were recorded. Unlike the *in vivo* data, significant differences in estradiol levels were observed between the exposed and control groups, these differences seemed to be linked to the stage of the estrous cycle at which the ovaries were removed from the animals. The authors suggest that differences in estradiol levels were not observed *in vivo* as homeostatic feedback from the hypothalamus-pituitary-ovarian axis maintaining circulating estradiol at normal levels. This is plausible, however clearly homeostatic mechanisms could not compensate for the reduction in progesterone levels.

Although it appears that there is considerable evidence that exposure to n-hexane or 2,5-HD leads to altered levels of endocrine hormones and adverse effects to the gonads and reproductive capacity of humans and laboratory animals, the mechanistic link between these two events is not yet clear. This is important in terms of the classification of these molecules as endocrine disruptors or reproductive toxicants. As stated above in the section interpreting the definition of an endocrine disruptor, it is widely accepted that a mechanistic link must be elucidated to confirm that any observed adverse physiological effects are the consequences of an alteration in the functioning of one or more endocrine axes. In the case of n-hexane and 2,5-HD, it is not clear whether the altered endocrine hormone levels are due to damage caused to the gonads, which would classify these molecules as reproductive toxicants, or whether the damage to the gonads is caused by altered hormone levels as postulated by the authors of several of these studies.

In the second case these molecules would be classified as endocrine disruptors. A 2005 US Environmental Protection Agency review of the toxicology of n-hexane appears to be in agreement with this conclusion, discussing the reproductive toxicity of n-hexane without mentioning endocrine disruption (https://cfpub.epa.gov/ncea/iris/iris_documents/documents/toxreviews/0486tr.pdf).

The observed morphological effects on the gonads of rodents (severe testicular atrophy and ovarian apoptosis) could easily be explained by an increased activity of the sex steroid axes. It has been well documented that increased circulating androgen levels either due to abuse of steroid hormones for muscle gain or by exposure to endocrine disruptors leads to testicular atrophy and disruption of female fecundity (Nieschlag and Vorona, 2015; Wolf *et al.*, 1999; Zaroogian *et al.*, 2001; Howe and Morello, 1985; Bilgi *et al.*, 2019).

Interestingly, despite the authors of several of the studies postulating that n-hexane and 2,5-HD are endocrine disruptors, the mechanisms identified for the reprotoxic effects of these molecules are listed as effects which do not seem to specifically target an endocrine axis, rather causing a toxic insult to the gonads. For example, a recent study found that "2,5-HD exposure disrupts hormonal homeostasis and induces oxidative stress in the ovary and uterus of rats", again failing to indicate the cause and effect relationship between these two events (Abolaji *et al.*, 2015; Adedara *et al.*, 2016). Interestingly, oxidative stress also seems to be the main mechanism of neuro, renal and hepatic toxicity for these molecules, suggesting that although certain organs are more sensitive than others, the initiating event may not be specifically endocrine (Adedara *et al.*, 2014; Qi *et al.*, 2019).

Less clear is whether the transgenerational effects observed in F1 adult female rats following exposure of their parents (F0 generation) to n-hexane would classify this chemical as an endocrine disruptor. These effects include altered expression of genes involved in ovarian hormone production which has been linked to their methylation status (Li *et al.*, 2015). At least in the F1 generation, the initial effects are hormonal and if severe enough to provoke adverse physiological outcomes, then this would be consistent with the definition of an endocrine disruptor.