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An overview of the available data on the reproductive toxicity of ethylene glycol

RIVM letter report 2023-0352
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Synopsis

An overview of the available data on the reproductive toxicity of ethylene glycol

The substance ethylene glycol is a precursor (source) which is used to make polyester fibres, polymers and polyethylene terephthalate (PET) resins. Ethylene glycol is also used for its antifreeze and cooling properties, for example as a substance in de-icing agents for aircraft windscreens. It is additionally used as a solvent in paint and as a plasticiser in plastics.

This literature overview was commissioned by the Health Council of the Netherlands. The Health Council will use RIVM's overview to assess whether ethylene glycol is toxic to reproduction and harmful to the health of unborn children. The Minister of Social Affairs and Employment has asked the Health Council for this advice.

RIVM has summarised the relevant scientific literature selected by the Health Council. RIVM has summarised a total of 29 studies in laboratory animals and cells.

Keywords: ethylene glycol, reproductive toxicity, reproduction, developmental toxicity, fertility

Publiekssamenvatting

Een overzicht van de beschikbare informatie over de schadelijke effecten van ethyleenglycol op de voortplanting

De stof ethyleenglycol wordt gebruikt om polyestervezels, polymeren en harsen van polyethyleentereftlaat (PET) te maken. Het wordt ook gebruikt omdat het antivries- en koeleigenschappen heeft, bijvoorbeeld als antivriesvloeistof voor de voorruiten van vliegtuigen. Verder wordt het bijvoorbeeld gebruikt als oplosmiddel in verf en als weekmaker in plastics.

Dit literatuuroverzicht is gemaakt in opdracht van de Gezondheidsraad. De Gezondheidsraad gebruikt het overzicht van het RIVM als startpunt om te beoordelen of ethyleenglycol gevaarlijk is voor de vruchtbaarheid en het ongeboren kind. De minister van Sociale Zaken en Werkgelegenheid (SZW) heeft de Gezondheidsraad om dit advies gevraagd.

Het RIVM heeft een samenvatting gemaakt van de relevante wetenschappelijke literatuur die is geselecteerd door de Gezondheidsraad. Het RIVM heeft de bevindingen van in totaal 29 studies in proefdieren en in cellen samengevat.

Kernwoorden: ethyleenglycol, reproductietoxiciteit, voortplanting, ontwikkelingstoxiciteit

Content

Summary

RIVM has summarised the available literature on the potential reproductive toxicity and developmental toxicity of ethylene glycol. Ethylene glycol is mostly used for the production of polyester fibres, polymers, and polyethylene terephthalate (PET) resins. It is also used for its antifreeze and coolant properties, for example as a de-icing fluid for windshields of aircrafts. Additionally, it can be used as solvent in paints and as a softening agent in the manufacture of plasticisers.

In the current report, a total of 29 studies were summarised. Of these, 6 were fertility studies, and 14 were developmental toxicity studies. The data found were summarised. At the request of the Dutch Minister of Social Affairs and Employment, the Health Council of the Netherlands will use the summaries to assess the reproduction toxic properties and to provide a recommendation for classification. Such an assessment was beyond the scope of the current report.

1 Introduction

The aim of current research is to identify and summarize the available data from studies with laboratory models, test animals and humans on the substance ethylene glycol. The focus of current literature review will be on the reproductive toxic properties of this substance.
At the request of the Dutch Minister of Social Affairs and Employment, the Health Council of the Netherlands will use the summaries to assess the reproductive toxic properties and to provide a recommendation for its classification. The assessment will be performed by the Health Council's Subcommittee on Classification of Reproductive Toxic Substances. This subcommittee falls under the Dutch Expert Committee on Occupational Safety, which focuses on health risks associated with occupational exposure of workers to chemicals.

Current RIVM report does not include an assessment of the reported reproductive toxic properties of ethylene glycol, nor does it provide a recommendation for classification of these substances based on the CLP-criteria (Regulation EC No 1272/2008).

The literature search which forms the basis of current literature overview is summarized in chapter 2. In chapter 3, the substance identification of ethylene glycol is provided. Chapter 4 presents information on international classifications of ethylene glycol. Available information on monitoring (i.e. environmental and biological exposure monitoring) and manufacture and use is presented in chapters 5 and 6, respectively. A summary of the (toxico)kinetics of ethylene glycol is described in chapter 7. Chapter 8 describes an overview of the data on reproductive toxicity: data on adverse effects on fertility, sexual function, development and on or via lactation.

2 Literature search

The Health Council of the Netherlands has performed a literature search using PubMed and Scopus. The literature search retrieved 1825 results of which 1773 were excluded. Only reproductive animal studies and a few *in vitro* studies were selected for an extensive summary by the Health Council. No relevant cohort studies, case-control studies, cross-sectional studies, or human experimental studies were available. Studies on ethylene glycol monomethyl ether, ethylene glycol monoethyl ether, ethylene glycol monobutyl ether, ethylene glycol monophenyl ether, ethylene glycol monohexyl ether and ethylene glycol diethyl ether were excluded by the RIVM as these were beyond the scope of the current literature summary.

Twenty animal studies, of which 6 were fertility studies and 14 were developmental studies, and 9 in vitro studies on cryopreservation were summarized. There were no relevant human studies available. RIVM also consulted the REACH registration dossier of ethylene glycol (publicly available on ECHA website)[1] and secondary sources, which included the ATSDR Toxicological profile for ethylene glycol (2010) [1] and the NTP monograph 2004 on ethylene glycol [2]. These were used to retrieve information on substance identification, classification, manufacture, monitoring and toxicokinetics.

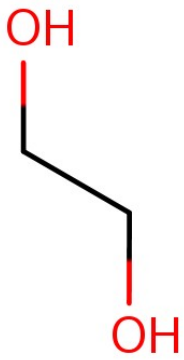
[1] https://echa.europa.eu/nl/registration-dossier/-/registered-dossier/15973

3 Substance identification

3.1 Name and other identifiers of the substance

The identity of ethylene glycol is presented in Table 1 below.

Table 1 Substance identity and information related to the molecular and structural formula of ethylene glycol.

Name(s) in the IUPAC nomenclature or other international chemical name(s)	**Ethylene glycol**
Other names (usual name, trade name, abbreviation)	Ethane-1,2-diol, 1,2-dihydroxyethane, 1,2-ethanediol, 1,2-ethylene glycol, 1-2 ethane-diol, 2-hydroxyethanol, ethylene alcohol, ethylene dihydrate, glycol, glycol alcohol, monoethylene glycol
ISO common name (if available and appropriate)	-
EC/EINECS number (if available and appropriate)	203-473-3
EC name (if available and appropriate)	Ethane-1,2-diol
CAS number	107-21-1
Other identity code (if available)	N/A
Molecular formula	$C_2H_6O_2$
Structural formula	
SMILES notation (if available)	OCCO
Molecular weight or molecular weight range	62.07
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	N/A
Description of the manufacturing process and identity of the source (for UVBC substances only)	N/A
Degree of purity (%) (if relevant for the entry in Annex VI)	N/A

N/A: Not applicable

3.2 Physico-chemical properties of ethylene glycol

The physico-chemical properties of ethylene glycol are presented in Table 2.

Table 2 Summary of physicochemical properties of ethylene glycol

Properties	**Value**	**Reference**
State of the substance at normal temperature and pressure	Colourless liquid (at 20°C and 1013 hPa)	[3]
Melting/freezing point	-13°C (at 1013 hPa)	[3]
Boiling point	197.4°C (at 1013 hPa)	[3]
Relative density	1.11 g/cm^3 (at 20°C)	[3]
Vapour pressure	12.3 Pa (at 25°C)	[3]
Surface tension	Not expected to be surface active	[3]
Water solubility	1000 g/L (at 20°C)	[3]
Partition coefficient n-octanol/water	-1.36 (at 25°C)	[3]
Flash point	111°C (at 1013 hPa)	[3]
Flammability	Non flammable	[3]
Explosive properties	Non explosive (100%)	[3]
Self-ignition temperature	398°C (at 1013 hPa)	[3]
Oxidising properties	Non oxidising (100%)	[3]
Granulometry	N/A	[3]
Stability in organic solvents and identity of relevant degradation products	N/A	[3]
Dissociation constant (pKa)	N/A	[3]
Viscosity	16.1 mPa·s (at 25°C)	[3]

N/A: Not applicable

4 International classifications

4.1 European Commission

Ethylene glycol has currently a harmonized classification in Annex VI of the CLP-Regulation (EC) 1272/2008 for:

- Acute tox. 4 (H302: harmful if swallowed)

4.2 The Health Council

Ethylene glycol has not previously been evaluated by the Health Council of the Netherlands.

4.3 Other countries

Ethylene glycol is not listed as carcinogen by the National Toxicology Program (NTP).[2]

In Germany, ethylene glycol is not included in the list of additional CMR substances in the context of worker protection.[3]

The state of California has included ethylene glycol in their safe drinking water and toxic enforcement act as a chemical known to the state to cause developmental toxicity after ingestion (since June 19, 2015).[4]

Ethylene glycol is currently not included in the list of substances NIOSH considers to be potential occupational carcinogens.[5]

Ethylene glycol has the following classification in Australia[6]:

- Acute tox. 4 (H302: harmful if swallowed)
- STOT SE. 3 (H335, may cause respiratory irritation)

Ethylene glycol has the following classification in Japan[7]:

- Acute tox. 5 (H303: may be harmful if swallowed)
- Skin corrosion/irritation 3 (H316: causes mild skin irritation)
- Serious eye damage/eye irritation 2B (H320: Causes eye irritation)
- Repro. 1B (H360: may damage fertility or the unborn child)
- STOT SE 1 (H370: causes damage to organs; central nervous system, kidney, heart, respiratory system)
- STOT RE 1 (H372: cause damage to organs through prolonged or repeated exposure; central nervous system, respiratory system, heart)

[2] https://ntp.niehs.nih.gov/ntp/roc/content/listed_substances_508.pdf
[3] https://www.baua.de/DE/Angebote/Rechtstexte-und-Technische-Regeln/Regelwerk/TRGS/pdf/TRGS-905.pdf?__blob=publicationFile
[4] https://oehha.ca.gov/media/downloads/proposition-65//p65list091319.pdf
[5] https://www.cdc.gov/niosh/topics/cancer/npotocca.html
[6] http://hcis.safeworkaustralia.gov.au/HazardousChemical/Details?chemicalID=1873
[7] https://www.nite.go.jp/chem/english/ghs/06-imcg-0094e.html

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5 Monitoring

5.1 Environmental exposure monitoring

Gas chromatography is the major technique used to measure ethylene glycol in environmental samples, such as air or water.

One method to measure ethylene glycol is included in the NIOSH Manual of Analytical Methods:

- Method 5523: Gas chromatography, flame ionized detection. This method is used for the analysis of several glycols in air samples, which can be identified and quantified using the gas chromatography parameters outlined in this method. This method replaces Method 5500 for the identification of ethylene glycol, which was found deficient in separating ethylene glycol from propylene glycol and for detection of ethylene glycol in aerosol form [4].

Two methods to measure ethylene glycol are included in EPA's SW-846 Compendium on hazardous waste test methods:

- Method 8015C: Gas chromatography, flame ionized detection. This method is used for the detection of several nonhalogenated volatile organic compounds and semi volatile organic compounds, including ethylene glycol, in water, by direct aqueous injection in a gas chromatograph [5].
- Method 8430: Gas chromatography, Fourier transform-infrared. This method is used for the detection of bis(2-chloroethyl)ether and its hydrolysis compounds, including ethylene glycol, in water, by direct aqueous injection into a gas chromatograph [6].

5.2 Biological exposure monitoring

Ethylene glycol can be measured in blood and urine by gas chromatography (GC) or high-performance liquid chromatography (HPLC). GC using either flame ionization detector (FD), alkali flame ionization or mass spectrometry (MS) for quantification. HPLC can be used to identify ethylene glycol and its metabolites glycolate, Hippurate and oxalate in biological samples such as urine and blood. Typically, a positive result using HPLC is confirmed by GC. Therefore, GC is the preferred method as HPLC could be omitted [1].

Scanning electron microscopy (SEM) can also be used to identify the metabolites of ethylene glycol in kidney tissue [7]. An enzymatic method has been developed in a hospital (DuPont Automated Clinical Analyzer triglyceride assay pack), to detect ethylene glycol in blood samples. However, this method is only applicable if concentrations are below 12 g/L and positive results must be confirmed by another method.

Table 3 Overview of methods of the analysis of ethylene glycol and metabolites in biological samples from ATSDR (2010)[1]

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery
Ethylene glycol (Human plasma)	Deproteinization with acetic acid, reaction with butyl-boronic acid, neutralize with NH4OH, extraction with dichloromethane	HRGC/MS	5 ppm	94-106
Ethylene glycol (Human serum)	Esterification with butylboronic acid and 2,2-dimethylpropane, neutralization with NH4OH in acetonitrile	HRGC/FID	NR	95
Ethylene glycol and glycolic acid (Human serum)	Supernatant treated with 2,2-dimethoxypropane/ dimethylformamide	GC/MS	10 ppm	91.1% (ethylene glycol) 77.6% (glycolic acid)
Ethylene glycol (Human serum)	Deproteinization using ultrafiltration.	CCGC	10 ppm	NR
Ethylene glycol (human serum and urine)	Derivatization with phenylboronate in methanol	HRGC/FID	1.0 ppm	90-98
Ethylene glycol (Human blood and tissue)	Derivatization with n-buylbornoic acid in acetone	GC/FID/AFID	NR	70
Glycolic acid (Human serum)	Colorimetric. Precipitation of protein with trichloroacetic acid, addition of chromotropic acid.	Absorbance at 580 nm or GC/FID	1.0 mmol/L (60 ppm)	NR
Glycolic acid (Human serum)	Extraction using methyl ethyl ketone, derivatization with PNBDI	HPLC/UV	0.05 mmol/L (3 ppm)	NR
Ethylene glycol (Urine)	Extraction with CHCL3	TLC	NR	NR
Sodium fluorescein (Urine)	Borosilicate tubes	Fluorescence (Wood's lamps)	NR	NR
Glycolic acid (Dog urine)	Extraction in MEK, dissolution of residue in ethylacetate, derivatization with PNBDI	HLPC/UV	1-2 ng	96
Oxalate (Human plasma and urine)	Deproteinated by addition of acetonitrile and phosphate buffer (blood), derivatization using	HPLC/UV	Plasma: 0.15 mg/L Urine: 0.5 mg/L	85

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery
	1,2-diaminoenzene (urine)			
Hippurate (Dog kidney tissue)	Ground with acidic methanol	TLC	NR	NR
Ethylene glycol (Human tissue)	Extraction with HPLC grade water	HPLC/RI	5 ppm	98 at 1 mg/mL

AFID = alkali flame ionization detector; CHCl3 = chloroform; CCGC = Capillary column gas chromatography; FID = flame ionization detector; GC = gas chromatography; HPLC = high-performance liquid chromatography; HRGC = high resolution gas chromatography; MEK = methylethyl ketone; MS = mass spectrometry; NH4OH = ammonium hydroxide; NR = not reported; PNBDl = O-p-nitrobenzyl-N,N'-diisopropylisourea; RI = refractive index detector; TLC = thin-layer chromatography; UV = ultraviolet detector

6 Manufacture and uses

Manufacture

Ethylene glycol also known as monoethylene glycol or glycol can be produced from three different processes.

The first process that can be used is an industrial production route from carbon monoxide (Figure 1 (a)). It is used in countries producing large quantities of coal. The oxidative carbonylation of methanol to Dimethyl oxalate provides an approach to the production of C1-based ethylene glycol. Furthermore, dimethyl oxalate can be converted into ethylene glycol in high yields (94.7%) by Hydrogenation.
Because the methanol is recycled, only carbon monoxide, hydrogen, and oxygen are consumed.

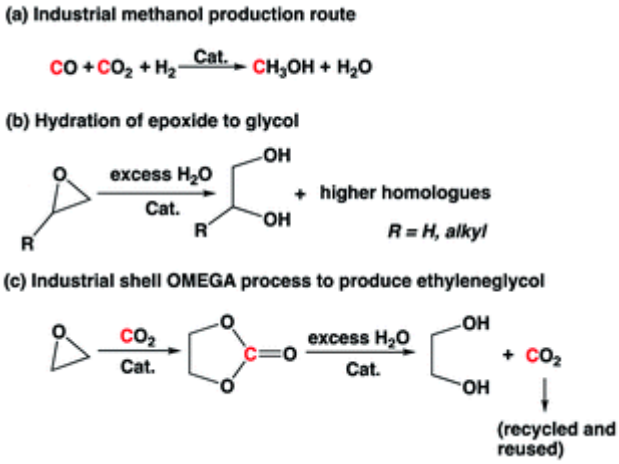

Figure 1 Industrial routes of production of ethylene glycol [8]

The second process is from ethylene oxide (Figure 1 (b)). The reaction with water allows the production of ethylene glycol in accordance with the following chemical equation:
$C_2H_4O + H_2O \rightarrow HO{-}CH_2CH_2{-}OH$
This reaction, catalysed by acids or bases, generates ethylene glycol with a yield of 90%. In addition, several by-products: oligomers diethylene glycol, triethylene glycol, and tetraethylene glycol.

Another method of producing ethylene glycol was developed by Shell. The equation is shown in Figure 1 (c). The ethylene oxide is first converted with carbon dioxide (CO_2) to ethylene carbonate [9]. The latter is then hydrolysed in a second step to produce ethylene glycol. The carbon dioxide is released in this step again and can be fed back into the process circuit. The carbon dioxide comes in part from ethylene oxide production, where a part of the ethylene is completely oxidized. According to Shell, the conversion efficiency of this process is over 99%.

Uses

Ethylene glycol is a substance used by consumers, in articles, by professional workers (widespread uses), in formulation or re-packing, at industrial sites and in manufacturing [3].

Ethylene glycol is mostly used for the production of polyester fibres, polymers, and polyethylene terephthalate (PET) resins. It is also used for its antifreeze and coolant properties like as a de-icing fluid for windshields and aircraft. Additionally, it can be used as a desiccant for natural gas production as well as in hydraulic brake fluids, electrolytic condensers, as solvents in paints and plastics, in inks and toners, washing & cleaning products, dyes, as a softening agent in cellophane, and in the manufacture of plasticisers, solvents, polishes and waxes, for non-metal-surface treatment products and other coating products, leather treatment products, adhesives and sealants, and biocides (e.g. disinfectants, pest control products) [1, 10, 11].

According to the REACH regulation, ethylene glycol is manufactured in and / or imported to the European Economic Area, at ≥ 1 000 000 to < 10 000 000 tonnes per annum [3].

Exposure

It is estimated that workers are exposed mostly through inhalation, skin and eyes. Inhalation is an occupational hazard for example for workers involved in airport de-icing operations by spraying the de-icing formulation through the air, generating ethylene glycol vapour and mist. Dermal exposure to ethylene glycol in de-icing fluids may also be important, especially for workers who do not have adequate skin protection during or after application [1].

7 (Toxico)kinetics

7.1 Summary

Below is a brief summary of the kinetics of ethylene glycol which is a direct copy of the factsheet of Public Health England in 2015 [12].

Ingestion and dermal exposure are the major routes of exposure to ethylene glycol, though dermal exposure is unlikely to lead to toxic effects. Following ingestion, ethylene glycol is readily absorbed throughout the gastrointestinal (GI) tract. Distribution is rapid and occurs throughout body water as indicated by a volume of distribution of approximately 0.7–0.8 L/kg. Peak concentrations following ingestion occur within 1–4 hours.

In humans and primates much of ethylene glycol's toxicity is mediated by its metabolites and not the parent molecule. The liver and kidneys are the primary site of metabolism for ethylene glycol, glycolic acid is its primary metabolite. Ethylene glycol is first oxidised by alcohol dehydrogenase to glycoaldehyde, which is then further metabolised to glycolic acid by mitochondrial aldehyde dehydrogenase and cytosolic aldehyde oxidase. Glycolic acid is then metabolised to glyoxylic acid by glycolic acid oxidase or lactate dehydrogenase. Glycolic acid oxidase also catalyses the formation of oxalic acid from glyoxylic acid. Glyoxylic acid may be metabolised to malate, formate or glycine. Lactic acid is also formed in the metabolic processes following ethylene glycol exposure. It is the accumulation of these acid products that accounts for much of the toxicity of ethylene glycol. Chelation of aqueous oxalic acid with calcium ions forms insoluble calcium oxalate, which cannot be further metabolised by humans.

Excretion of ethylene glycol is primarily in the urine either as the parent molecule, glycolic acid, calcium oxalate or glycine (and its conjugate hippurate). Oxalic acid is excreted in the urine and may give rise to dihydrate and or monohydrate oxalate crystals which may precipitate in the kidney causing nephrotoxicity. Approximately 20% of a dose of ethylene glycol may be excreted unchanged by the kidneys. The elimination half-life in humans is estimated to be in the range of 2.5–8.4 hours.

7.2 Human data

The following paragraph is a summary of the human data in the toxicokinetics chapter of the toxicological profile of ethylene glycol written by the Agency for Toxic Substances and Disease Registry (ATSDR) in 2010 [1].

7.2.1 Absorption

Limited information indicates absorption via inhalation. Two studies with both two human volunteers using radiolabelled ethylene glycol as well as one case report show internal exposure after inhalation exposure. After acute oral poisoning with ethylene glycol, internal exposure was observed but the rate of absorption cannot be quantified. A study with

three volunteers indicates dermal absorption of approximately 1% of the dermal applied dose.

7.2.2 Distribution

Estimated volume of distribution (Vd) after inhalation exposure was 0.78 and 0.91 L/kg in two volunteers. After oral exposure, ethylene glycol distributes according to total body water with an apparent Vd of 0.54-0.56 L/kg based on data of two poisoned patients.

7.2.3 Metabolism

The metabolism of ethylene glycol was reviewed by the NTP-CERHR in 2004 and Slikker et al. [1, 2, 13]. Figure 2 provides an overview of the metabolism and is based on these two reviews. Briefly, the main metabolic pathway is as follows. Ethylene glycol is metabolised by alcohol dehydrogenase to glycoaldehyde, which is a process that can be saturated and is therefore a rate-limiting step. Glycoaldehyde is rapidly converted to glycolic acid by aldehyde dehydrogenase which is then oxidized to glycoxylic acid by glycolic acid oxidase or lactic dehydrogenase. This oxidation is the major rate-limiting step in the metabolism. Glyoxylic acid can be metabolized to create glycine, or malate, all of which may be further broken down to generate respiratory CO_2, or to oxalic acid, which is excreted in the urine. In excess, oxalic acid can form calcium oxalate crystals. Both glycolic acid and oxalic acid are also products of normal protein and carbohydrate metabolism.

In volunteers who inhaled radiolabelled ethylene glycol for 4h, glycolic acid concentrations peaked at 4-5h after exposure. About 1% of the estimated dose was excreted as glycolic acid and 0.08-0,28% was excreted as oxalic acid over 30h. In eight intoxications, plasma glycolate levels ranged from about 12 to 29 mM.

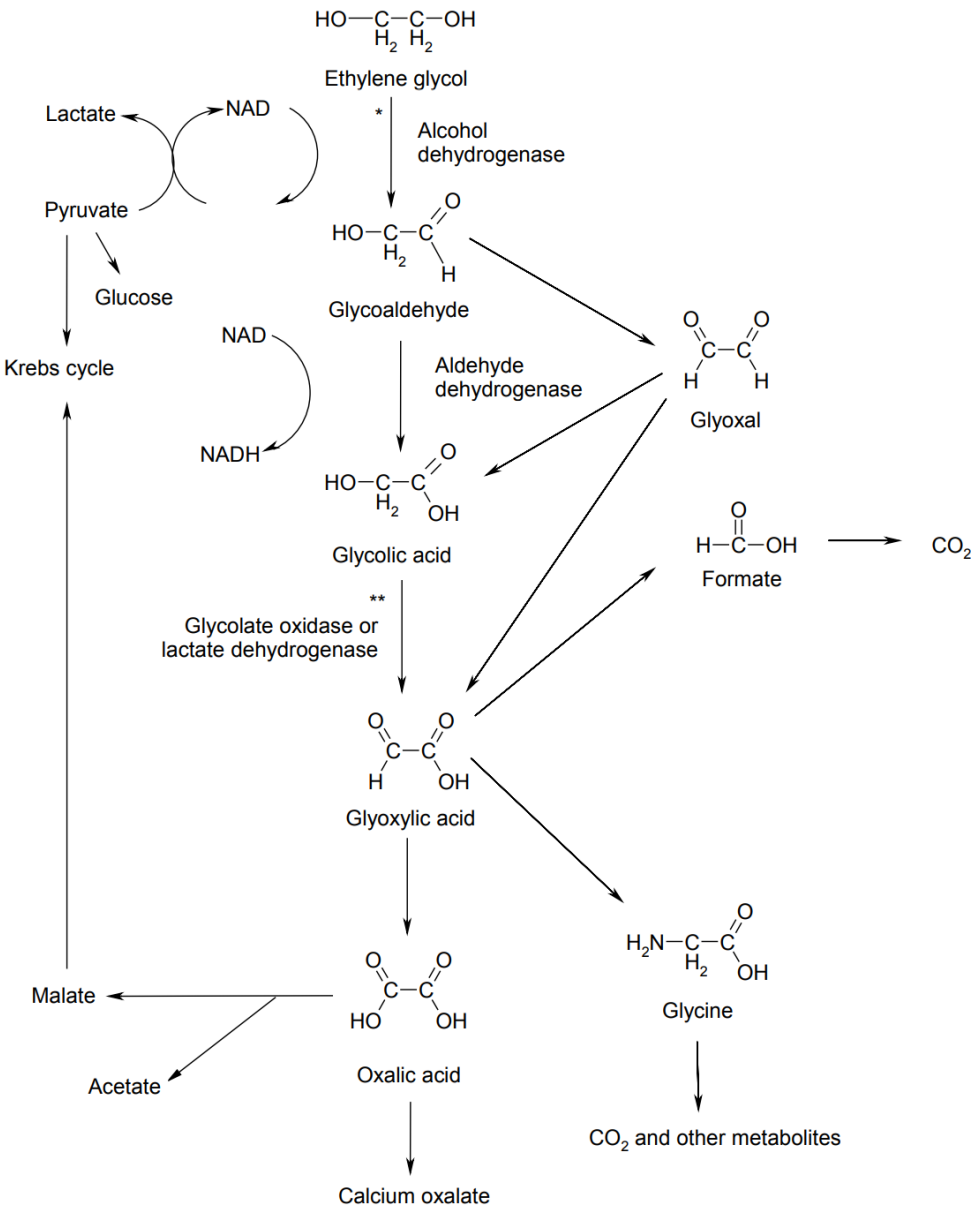

Figure 2 Metabolic pathway for ethylene glycol (source: [1, 2, 13])
* Rate-limiting step; **Most rate-limiting step

7.2.4 Elimination

In two volunteers inhaling radiolabelled ethylene glycol at estimated doses of 0.96 and 1.51 mg/kg, urinary excretion up to 30h after exposure of ethylene glycol was 6.4-9.3% of the dose and the sum of glycolic acid and oxalic acid was 1-2% of the inhaled dose. Ethylene glycol or metabolites in exhaled air were not detected but CO_2 was not measured and all concentration estimations were highly uncertain according to the ATSDR. Similar results were obtained in two other volunteers.

In untreated adults, serum half-life of ethylene glycol is between 3 and 8.4h. The rate of ethylene glycol elimination is greatly increased by dialysis to 1.5-3h indicated by two studies in patients.

7.3 Animal data

The following paragraph is a summary of the human data in the toxicokinetics chapter of the toxicological profile of ethylene glycol written by the Agency for Toxic Substances and Disease Registry (ATSDR) in 2010 [1].

7.3.1 Absorption

In rats exposed nose-only to radiolabelled ethylene glycol, the estimated absorption was 60-90% of the initial dose. Oral absorption is rapid and near 100%. In rats peak blood levels are reached within 1h and in mice, monkeys dogs and pregnant rabbits between 1-3h. Pregnancy does not alter kinetics in rats. A single study indicates apparent dermal absorption of 26-32% and 60-84% of the applied dose in rat and mice respectively.

7.3.2 Distribution

In rats that inhaled radiolabelled ethylene glycol, 75-80% of the initial body burden was distributed quickly throughout the body and it was estimated that 60% ends up in the respiratory tract (mainly nasal cavity). In rats and mice, 96h after a single radiolabelled dose, 6-22% and 3-11% of the radioactivity was retained in tissues for rat and mice, respectively. Highest radioactivity was found in the liver of both species. In two rhesus monkeys unmetabolized ethylene glycol was evenly distributed throughout tissues (tissue-to-plasma ratios of 0.85-1.91).

Ethylene glycol crosses the placenta and enters the developing foetus in rats and rabbits after oral administration to pregnant dams. Unchanged ethylene glycol in yolk sac and embryos were 14-20% of maternal concentrations in a toxicokinetic study in rabbits. Extraembryonic fluid levels of ethylene glycol were 2-fold higher in rats than in rabbits after a single oral administration at GD10.

After dermal exposure, levels of ethylene glycol are the highest in pelt, namely 5-6% of the applied dose or 8-15% of the applied dose when carcass and pelt are combined.

7.3.3 Metabolism

Glycolic acid was the major metabolite in the plasma of orally exposed male rats (single dose). During 12h postdosing, at 100 and 1000 mg/kg, glyoxylate and glyoxal as well as trace levels of glycoaldehyde were detected and at 10 mg/kg glyoxylate levels exceeded glycolate levels. Plasma glycolate levels peaked 6h or 4h after a single dose in rats or dogs respectively where ethylene glycol peaked after 2h in both species.

Enzymes metabolizing glycolate are more quickly saturated with bolus subcutaneous dosing than with slow, continuous dosing, leading to higher peak plasma glycolate levels of 3-10 fold with bolus dosing.

Multiple *in vivo* studies in rats and mice show increasing urinary excretion of glycolic acid and other metabolites with increasing dose, probably corresponding to saturation of glycolic acid metabolism. In male rats after a single radiolabelled oral dose (1000 mg/kg), glycolic acid comprised 25% of the urinary radioactivity in the first 12h. In the

following 12h, this amounted to 37% and oxalic acid was detected at 7.4%. At low dose (10 mg/kg), >90% of the urinary radioactivity was unmetabolized ethylene glycol. The metabolism pattern was similar in female rats. In mice however, only glycolate was detected with increasing excretion at higher doses. Urinary excretion of ethylene glycol and glycolate accounted for 20.7 and 4.5% respectively, of a 2000 mg/kg dose in rats during 24h post-dosing.

In percutaneously exposed rats and mice to radiolabelled ethylene glycol (10 or 1000 mg/kg) most of the radioactivity was excreted as ethylene glycol. In rats this was 87-100% regardless of the dose, but in mice glycolate represented a greater proportion of urinary radioactivity at 1000 mg/kg (up to 20%).

Male porton rats exposed to ±1000 mg/kg ethylene glycol in drinking water for 21 days had urinary oxalate levels equivalent to 1.18% conversion of ethylene glycol to oxalate. In another study, male Wistar rats were treated with ethylene glycol up 10 400 mg/kg/day for 12 months. From >300 mg/kg/day, glycolic acid levels in the kidney, blood and urine were increased compared to control. Oxalate levels were increased at >300 mg/kg/day in the kidney but were similar across all doses in blood and urine. It is suggested that the metabolism of glycolic acid occurs between 150 and 300 mg/kg/day in Wistar rats.

In vitro data from one study in liver homogenates and a study using liver slices both suggest that human may metabolize glycolic acid at a higher rate than rats (approximately 2-4 fold higher Vmax/Km values in human tissues). According to the NTP-CERHR, *in vivo* human data to predict the saturation point in humans were lacking [2].

One study suggests that pregnancy does not alter the pharmacokinetics in maternal blood and urine when groups of pregnant and nonpregnant rats were treated with a single dose at GD10 (for the pregnant rats). However, a single time point is considered a too narrow exposure window to observe pregnancy-related changes in metabolism. Foetal levels of ethylene glycol or metabolites were not measured. In another study in rats, levels of glycolic acid in embryos and extraembryonic fluid paralleled maternal levels but were 1.4-4 fold higher than maternal levels. It is reported that there are species-specific differences in the transfer of glycolic acid from maternal blood to conceptus.

7.3.4 Elimination

In laboratory animals treated with radiolabelled ethylene glycol the primary routes of excretion are exhaled air and urine, regardless of the exposure route. After oral exposure, saturation of metabolic pathways at higher doses leads to a greater urinary excretion and decreased elimination via air.

Rats exposed to ethylene glycol vapour (32 mg/m^3, 30 min) or aerosol (184 mg/m^3, 17 min) excreted 63% (over 4 days) or 75% (over 6 days) as CO_2. Urinary excretion was 20 or 12% of the initial body burden after vapour and aerosol exposure respectively, while faecal excretion was 3% and 1%.

Elimination half-lives in plasma of laboratory animals after oral exposure at 1.4-2.5h in rats, 0.3-1.1h in mice, 3.5h in dog and 2.7-3.7h in monkeys at multiple dose levels all including the 1000 mg/kg level. Data from intravenously exposed animals show similar half-lives. Plasma elimination half-life in pregnant rats was similar to regular rats.

In male and female rats receiving a single oral dose between 10 and 1000 mg/kg, the major excretory routes up to 96h of exposure were CO_2 exhalation (27-48% of radioactivity), urine (21-43%) and faeces (2-4%). Female mice that were exposed similarly as rats, excreted ethylene glycol as exhaled CO_2 (22-55%) and exhaled volatile organic compounds (3-11%), while excreting 24-56% via urine and 5-6% in faeces. The majority was eliminated within 12h after dosing. An increase in urinary excretion was evident between 10-100 mg/kg in female mice, between 10 and 400 mg/kg in female rats and between 800 and 1000 mg/kg in male mice. This probably resulted from saturation of enzymes that metabolize glycolic acid leading to increased excretion of this metabolite via urine.

Monkeys given a single oral dose (±1000 mg/kg), excreted 24% of the dose as the parent compound in urine within 48h. Dogs excreted about 50% of a single administered oral dose (173 mmol/kg) via urine within 72h.

Rats and mice were treated with dermal application of 10-1000 mg/kg undiluted or 1000 mg/kg 50% aqueous solution of radiolabelled ethylene glycol under occlusion for 6h. Measured for 96h after dosing, rats expired 6-14% of the administered dose, 4-8% was excreted in urine and 1% in faeces. Female mice exhaled volatile organic compounds (21-34%) and CO_2 (10-16%). No dose-related shift in excretory patterns was observed suggesting that metabolic pathways were not saturated with dermal exposure.

8 Toxicity on reproduction

8.1 Adverse effect on sexual function and fertility

The reproductive studies of ethylene glycol in experimental animal studies are summarized in Table 4 followed by a summary in text. In general, only statistically significant results are presented in the table below. In studies where statistical significance of the results was not present, effects that were reported by the authors are presented in the table below.

Table 4 Oral studies on ethylene glycol

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Lamb, 1985 [14]	COBS Crl:CD1, (ICR)BR outbred albino mice (CD1 mice) N=20/sex/group Controls: 40/sex	Reproductive study (non-guideline, non-GLP). Fertility assessment by continuous breeding (NTP protocol) with a preparatory dose range finding study. <u>Design:</u> - Premating period of 7 weeks - 98 days cohabitation and breeding (male+female), all pups born during this period were killed - 21 days segregation period, pups born during this period were kept alive - Exposure occurred during premating, cohabitation, segregation and throughout the life of offspring. - High dose and control pups (N=20/sex/group) born during segregation were mated.	Purity: 99.6% <u>Concentrations:</u> 0, 0.25, 0.5 and 1.0% (w/v), oral via drinking water. According to the authors, this is equal to 410, 840 and 1640 mg/kg bw/day, based on daily water consumption and mean body weight data. <u>F1 parents:</u> 0, 1.0% (w/v), oral via drinking water. This is equal to 1640 mg/kg bw/day, based on daily water consumption and mean body weight data.	<u>P0 Parents</u> No general toxicity in parental animals, i.e. clinical signs, effects on body weight or water consumption. Some deaths occurred in all groups. One death in the 0.5% group could be treatment related according to the authors (oxalate crystals in renal tubules). <u>F1 parents</u> No general toxicity observed.	<u>P0 parents:</u> - Decreased number of litters per fertile pair at high dose (4.5±0.2) versus control (4.9±0.08), P<0.01. - Decreased mean number of live pups per litter high dose (10.2±0.3) versus control (10.8±0.5), P<0.05. <u>F1 parents:</u> - Fertility was lower in high dose animals (61%) compared to controls (81%), not significant. - Number of live pups per litter and live pup weights were lower in high dose animals (not significant).	The authors measured drinking water consumption per pair of mice for 2 weeks. This could be used to calculate actual dose levels.

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
		Examinations: - Fertility and reproductive performance of the adults and F1 generation. Viability, sex and weight of the pups. - Organ weights of F1 offspring and skeletal examination of a proportion of F1 mice. - Histology of the head of F1 mice. Statistics: - Results presented as Mean ±SE. - Tests used differ per parameter. Mostly Chi-Square approximation to Kruskall Wallis is used for group comparisons and Mann-Withney U or Fisher exact test for pairwise comparisons. An ANOVA was carried out for assessment of body weight.	Dosage solutions were within 98 – 107% of intended concentrations.			
Gulati, 1986 [15]	Crl:CD-l (ICR)BR outbred	Reproductive study (non-guideline, GLP-compliant). Fertility	Purity: 99.6% Concentrations:	P0 parents: - No treatment-related effects on body, liver or	P0 parents - 1.5% - increased abnormal sperm counts	Follow-up from the

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
	albino mice (CD1) N=20/sex/group Controls: 40/sex	assessment by continuous breeding <u>Design:</u> - Premating period of 7 weeks - 98 days cohabitation and breeding (male+female), all pups born during this period were killed - 21 days segregation period, pups born during this period were kept alive - Exposure occurred during premating, cohabitation, segregation and throughout the life of offspring. - Pups from all dose groups (N=20/sex/group) born during segregation were mated. <u>Examinations:</u> - Fertility, viability and weight of litters.	0, 0.5, 1.0, 1.5% (w/v), oral in drinking water. According to the authors, this is equal to 0, 897, 1798 and 2826 mg/kg bw/day, based on daily water consumption and mean body weight data. Dosage solutions were within 94 and 104% of intended concentration	kidney weight observed in female mice. - Significant reduction in body weight (42.287±0.995 g in 1.5% group vs 46.449±1.036 g in control group) and liver weight (1.933±0.052 g in 1.5% group vs 2.113±0.066 g in control group) observed in male mice. <u>F1 parents:</u> 1.5% ethylene glycol resulted in statistically significant (P<0.05) reduced liver weight in females (1.518±0.050 g, vs 1.652±0.051 g in control groups) and males (1.692±0.051 g vs 1.869±051 g).	(8.28±2.02 vs 5.06±0.53 in control groups, P<0.05). - Reduced sperm mobility (80.6±8.05 vs 94.3±0.51 in control group, P<0.05) - Reduced live pup count - Non-significant reduction in fertility (14/16 in 1.5% ethylene glycol group vs 38/38 in control group). <u>F1 parents – 0.5%</u> **-** Reduced testes weight (0.124±0.006 g vs 0.140±0.005 g in control group, P<0.05) - Reduced sperm density (801±61 compared to 1036±63 in control group, P<0.05) <u>F1 parents – 1.0%</u> **-** Reduced testes weight (0.119±0.004 g vs 0.140±0.005 g in control group, P<0.05) - Reduced sperm density (855±63) compared to 1036±63 in control group, P<0.05) - Reduced sperm motility (92.1±1.47 vs 94.6±0.89 in control groups, P<0.05).	study by Lamb et al. (1985), higher dose tested.

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
		- Organ weight and skeletal deformities in offspring. <u>Statistics:</u> Data is displayed as mean ±SE. Cochran-Armitage or Jonckheere test for trends. Statistics differ per parameter, most used are Chi-Square, Kruskal-Wallis, Wilcoxon rank-sum and Fisher's exact test.			<u>F1 parents – 1.5%</u> **-** Reduced testes weight (0.120±0.006 g vs 0.140±0.005 g in control group, P<0.05) - Reduced sperm motility (84.1±5.02 vs 94.6±0.89 in control groups, P<0.05).	
DePass, 1986 [16]	Fisher 344 Rats N= 10 males/ group and 20 females/ group	Three-generation reproduction study (non-guideline, non-GLP). <u>Design:</u> - Premating period of 7 weeks - During breeding, period not mentioned. - Offspring was exposed throughout life and started breeding on 100 days of age. <u>Examinations:</u>	Purity: 99.93% <u>Concentrations:</u> Approximately 0.04, 0.2 and 1.0 g/kg bw/day in diet. Two untreated control groups.	No effects on mortality, diet consumption or body weight observed.	No treatment related effects on fertility index, gestation index, gestation survival index or days from first mating to litter observed.	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
		Fertility, behaviour and histopathology, kidney lesions. Statistics: continuous data using Barlett's test. T-test for equal and unequal variance. Frequency data using chi-square and Fisher's exact tests.				
Hong, 1988 [17]	B6C3F1 mice, males and females N=7/sex/group	4-day mouse study (non-guideline, non-GLP). Histological examinations on day 1 post exposure: lung, heart, liver, kidneys, adrenal glands, spleen, thymus, stomach, bone marrow, urinary bladder, small and large intestine, uterus (female) or testes (male). Statistics: Wilk-Shapiro test for normality, one-way analysis of variance and Dunnett's test for	Purity: 99.6% Concentrations: 0, 50, 100 or 250 mg/kg bw/day by oral gavage. Positive control: Ethylene glycol monomethyl ether, 0, 50, 100 or 250 mg/kg bw/day by oral gavage.	No mortality observed. Significant reduction in body weight for female mice ($P<0.01$) observed in 250 mg/kg bw/day group (21.4±0.3 g vs 23.4±0.2 g in control groups). No histological evidence of organ or tissue damage observed.	No effect on testicular weight observed in male mice. Positive control: Significant ($P<0.01$) decreases in testicular weight in 250 mg/kg bw/day group (3.3±0.1 g vs 4.1±0.2 g in control group)	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
		multiple comparison with control group.				
Harris, 1992 [18]	Crl:CD-1 mice N=10/sex/group	Short-Term Reproductive and Developmental Toxicity Screening study (non-guideline, non-GLP). <u>Design 1:</u> - Treatment of pregnant female mice during GD8-14 <u>Design 2:</u> - Treatment of males (SD3-20) and females (SD0-20) - Cohabitation on SD8 - Animals are sacrificed on study day 21. <u>Examinations:</u> - Liver, kidney and testes histology in male mice. - Fertility and litter weight. <u>Statistics:</u> Cochran-Armitage test for linear trend followed by Fisher's exact test.	Purity not mentioned. <u>Concentrations:</u> 0, 250, 700, and 2500 mg/kg/day in drinking water. This is equivalent to 0, 50, 140 and 500 mg/kg bw/day conform the CLP guidance [19]. Positive control: Ethylene glycol monomethyl ether, 0, 70, 250, and 700 mg/kg/day in drinking water. This is equivalent to 0,14, 50, and 140 mg/kg bw/day conform the CLP guidance [19].	No adverse clinical signs observed. No mortality observed.	<u>Males:</u> no effects on testes weight, sperm mobility or number observed. <u>Females (study design 1):</u> - No effects on numbers of live or dead implants observed. <u>Females (study design 2):</u> - 2500 mg/kg/day continuous exposure resulted in a significant increase in dead (1.4±0.4 vs 0.4±0.2) and a significant decrease in live (7.4±1.2 vs 10.0±0.9 in control groups) uterine implants ($P<0.05$). - No effects on fertility observed. <u>Positive control:</u> - Reduction in testes weight, sperm mobility and number ($P<0.05$) in 250 and 700 mg/kg/day exposure groups. - Study design 1: reduced number of live implants ($P<0.01$) in 250 and 700 mg/kg/day groups.	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
		Kruskal-Wallis analysis of variance for dose group comparison and Jonckheere's test for dose-responses. Mann-Whitney U test for pairwise comparisons.	Dosage solutions were between 93-100% of target concentrations.		- Study design 2: reduced impregnation (P<0.05) in 700 mg/kg/day group.	
Bolon, 1997 [20]	Crl:CD-l (ICR)BR outbred albino mice (CD1) N=20/sex/group Controls: 40/sex	Retrospective study (non-guideline, GLP compliant), ovaries obtained from 18 Reproductive Assessment by Continuous Breeding (RACB) bioassays in NTP archive. Design: - Premating period of 7 weeks - 98 days cohabitation and breeding (male+female), all pups born during this period were killed - 21 days segregation period, pups born during this period were kept alive - Exposure occurred during premating, cohabitation, segregation	Concentrations: Parental: 0%, 1.5% (w/v), oral via drinking water. This is equivalent to 0 and 3000 mg/kg bw/day conform the CLP guidance [19]. Offspring: 0%, 0.5%, 1%, 1.5% (weight/volume), oral via drinking water. This is equivalent to 0, 1000, 2000 and 3000 mg/kg bw/day conform the CLP guidance [19].	Not mentioned.	Parental animals: Non-significant increase in ovarian follicle count in 1.5% group. Offspring: 0.5%, 1% and 1.5% ethylene glycol exposures resulted in non-significant reductions in number of small, growing and antral ovarian follicles observed versus control. Positive control: - Parental: Non-significant reduction in ovarian follicle counts. - Offspring: significant reduction (P<0.05) in small, growing and antral ovarian follicle counts compared to controls.	This study compared several chemicals. Data on ethylene glycol are included in the tables, but ethylene glycol is not further discussed in the text of this paper.

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
		and throughout the life of offspring. - High dose and control pups (N=20/sex/group) born during segregation were mated. Examinations: Ovarian follicle counts (N=10) Statistics: Group comparisons using Kruskal-Wallis non-parametric ANOVA and dose response trends using Mann-Witney U test.	Purity not mentioned. Positive control: ethylene glycol monomethyl ether, 0, 0.03, 0.1, 0.2 and 0.3% (w/v) oral via drinking water.			

Lamb, 1985 [14]
A non-guideline fertility assessment by continuous breeding was performed by Lamb et al. in 1985. CD-1 mice (20/sex/group) were dosed at 0.25, 0.5, and 1% ethylene glycol in drinking water. A control group of 40/sex was present. Doses are equal to 0, 410, 840 and 1640 mg/kg bw/day, based on daily water consumption and mean body weight data. Exposure occurred during a premating period (7 weeks), a male/female cohabitation and breeding period (98 days), a segregation period (21 days) and throughout the life of the offspring. High dose and control pups that were born during the segregation period (20/sex/group) were mated to assess fertility of the offspring.
No general toxicity occurred in the parental animals, but some deaths occurred in all groups. According to the authors, one death in the 0.5% group could be treatment-related as oxalate crystals in the renal tubules were found. Some fertility effects in parental animals were found. At the highest dose, there were decreased number of litters per fertile pair in F0 parents. The fertility of the F1 parents was 20% lower compared to controls but this was not statistically significant.

Gulati, 1986 [15]
A non-guideline continuous breeding study, as follow-up of Lamb et al. (1985), was performed by Gulati et al. (1986). CD-1 mice (20/sex/group) were dosed at 0.5, 1.0, and 1.5% ethylene glycol in drinking water, 0% controls were included (40/sex), equal to 0, 897, 1798 and 2826 mg/kg bw/day, based on daily water consumption and mean body weight data. Mice were exposed during the premating period (7 weeks), during the breeding period (98 days), the segregation period (21 days) and throughout the life of the offspring. Offspring in all dose groups were mated (20/sex/group). Significant reduction in body weight and liver weight were observed in P0 parental male mice. No effects on body weight or treatment-related lesions in kidney, liver, ovary, uterus, or vagina were observed in female mice. In the group exposed to 1.5% ethylene glycol, a non-significant reduction in fertility and a significant reduction in litter size, live pups and sperm mobility was observed. In F1 mice, 1.5% ethylene glycol exposure showed a significant reduction in liver weight, in both female and male mice, and a significant reduction in testes weight in male mice. The groups exposed to 0.5% and 1% ethylene glycol showed a significant reduction in sperm density and the 1% and 1.5% dose groups showed a significant reduction in sperm mobility in F1 mice.

DePass, 1986 [16]
A non-guideline three generation reproduction study was performed by DePass et al. (1986). Fisher 344 Rats (10 males and 20 females/group) were treated with 0.04, 0.2, and 1.0 g/kg/day ethylene glycol (purity >99.9%) in diet. Rats were exposed during a 7-week premating period and throughout breeding. Offspring (10 males and 20 females/group randomly chosen from the litters) were exposed throughout life and breeding was started at 100 days of age. No treatment related effects on mortality, diet or body weight changes were observed. No treatment related effects on fertility index, gestation index, gestation survival index or days from first mating to litter were observed for all three generations. Effects on developmental toxicity described within this study are discussed in section 8.2.

Hong, 1988 [17]
A non-guideline mouse study was performed with male and female B6C3F1 mice. Ethylene glycol (purity 99.6%) was administered for 4 consecutive days via oral gavage in water at doses of 0, 200, 400, and 1000 mg/kg bw/day (7/sex/group). Statistically significant reduction in body weight was only observed for female mice in the 1000 mg/kg bw/day group. No histological evidence of organ or tissue damage and no significant effects on testicular weight were observed. This study also tested ethylene glycol monomethyl ester, which showed a statistically significant reduction of testicular weight and mild to moderate segmental degeneration of seminiferous tubules in testes of male mice in the 1000 mg/kg bw/day group.

Harris, 1992 [18]
A non-guideline 21-day developmental toxicity study was performed by Harris et al. (1992). CD-1 mice (10/sex/group) were exposed to 0, 250, 700, and 2500 mg/kg/day ethylene glycol in drinking water. Females were exposed between gestation days 8 and 14, or continuously for 21 days from study day 0. Males were exposed from study day 3. Cohabitation and breeding started on study day 8 and males were killed on day 20 and females on day 21. No treatment related effects on testes weight, sperm number or mobility were observed in male mice. Exposure between gestation days 8 and 14 did not result in adverse clinical signs or effects on the number of live or dead implants in females. 2500 mg/kg/day continuous exposure resulted in a significant increase in dead and a significant decrease in life uterine implants in female mice. Ethylene glycol monomethyl ester exposure resulted in statistically significant reduced tested weight, sperm mobility and number. Exposure during gestation resulted in a reduced number of live implants and continuous exposure resulted in reduced impregnation in the 700 mg/kg/day dose groups.

Bolon, 1997 [20]
A non-guideline retrospective study was performed by Bolon et al. (1997) on the ovaries of mice from 18 bioassays. CD1 mice (20/sex/group) were dosed at 1% ethylene glycol in drinking water, 0% controls were included (40/sex). Mice were exposed during the premating period (7 weeks), during the breeding period (98 days), the segregation period (21 days) and throughout the life of the offspring. Offspring (20/sex/group) was dosed with 0.5%, 1% or 1.5% ethylene glycol in drinking water. General toxicity parameters are not stated. A non-significant increase in ovarian follicle counts upon 1% ethylene glycol exposure was observed for the parental group compared to the control. A non-significant decrease in ovarian follicle count was observed in the 0.5%, 1%, and 1.5% ethylene glycol exposure groups compared to the control in offspring.

8.2 Adverse effects on development

The developmental studies of ethylene glycol in experimental animal studies are summarized in Table 5 followed by a summary in text. Only statistically significant results are presented, unless specified otherwise. In some studies, statistical significance of certain findings was not presented by the authors. In such a case, the effects that were reported by the authors are presented in the table below without mentioning a P-value.

Table 5 Oral studies on ethylene glycol

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rats						
Maronpot, 1983 [21]	Rats, Fischer 344, females. N=20/group	Prenatal development study (non-guideline, non-GLP). Design: Treatment of pregnant dams from GD 6 to 12. C-section on GD 21. Examinations: visceral (nasal cavity, eyes, brain) and skeletal alterations and malformations. Statistics: - Continuous data: F-test (with paired group F-max test or t-test) - Non-parametric data: multiple sum of ranks test. - Binominal data: Fischer exact test	Purity: >99.9% Concentrations: 0, 40, 200 and 1000 mg/kg bw/day (targeted dose) in diet. Negative controls: regular diet. Positive control: hydroxyurea 500 mg/kg (ip) on GD11.	No effect on body weight gain and no clinical signs.	1000 mg/kg bw/day: Preimplantation loss was higher (median: 23.0% IQR: 9.0-42.0%) compared to control (median: 10.0% IQR: 2.0-29.2%) but not statistically significant. 1000 mg/kg bw/day: - Increased incidence of poorly ossified (14.2% versus 1.8%, P<0.001) and unossified vertebral centra (26.0% versus 11.4%, P<0.001) compared to control. Major malformations did occur in the positive controls.	
Price, 1985 [22]	Rat, CD, females N=>10/group/replicate	Developmental study (NTP study, GLP compliant).	Purity: >99% Vehicle: water Concentrations:	Maternal toxicity: Piloerection in all treated groups but not in controls.	Developmental toxicity: - Increased post-implantation loss per litter at the high dose	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rats						
	(so total of 20/group)	Design: Treatment of pregnant rats from GD6 through 15. Two replicates of the teratology evaluation were conducted. Caesarean section was performed on GD 20. Examinations: maternal organ weights, uterine contents and visceral, skeletal and morphological abnormalities. Statistics: - Data are presented as mean ± SEM - Dose response: test for linear trend - ANOVA with post hoc tests - test for linear trend on proportions and chi-square test (nominal data). One tailed- fisher	0, 1250 (low), 2500 (mid) or 5000 (high) mg/kg bw/day, via oral gavage Negative control: vehicle Actual dose levels were within 10% of the calculated levels.	- Dose related decrease in all maternal body weight parameters and gravid uterine weight. - Decreased weight gain during treatment (all P<0.01): Control: 42.03±1.96g 1250 mg/kg: 34.81±1.73g 2500 mg/kg: 29.45±1.38g 5000 mg/kg: 20.68±1.93g - Decreased weight on GD20 (mid and high dose, P<0.01): Control: 366.06±3.94g 2500 mg/kg: 345.69±5.86g 5000 mg/kg: 324.99±6.64g - Decreased weight gain during gestation (mid and high dose, P<0.01): Control: 129.50±3.06g	(21.34± 5.24%) compared to control (4.70± 1.23%), P<0.05. - Decreased number of live foetuses per litter at mid dose (11.90±0.60) and high dose (11.04±0.79), P<0.05. - Decreased average foetal body weight per litter at mid dose (2.916±0.056 g) and high dose (2.388±0.089 g) compared to control (3.404±0.052 g). - Increase in percentage live foetuses malformed per litter at mid dose (25.11±4.84%) and high dose (75.53±6.42%) compared to control (1.37±0.97%). - Increase in percentage litters with malformed live foetuses at low dose (39.29%, P<0.01), mid dose (68.97%, P<0.001) and high dose (96.15%, P<0.001). Morphologic defects: Dose response relationship observed for all types of malformations. -External malformations in 15 litters at high dose compared to 0	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rats						
		exact test for pairwise comparison.		2500 mg/kg: 108.26±4.06g 5000 mg/kg: 90.17±6.35g - Decreased gravid uterine weight (mid and high dose, (P<0.01): Control: 73.04±1.63g 2500 mg/kg: 58.00±2.97g 5000 mg/kg: 46.61±3.75g - Decreased liver weight (high dose, P<0.05): Control: 15.47±0.26g 5000 mg/kg: 13.70±0.35g - Relative kidney weight increased (mid and high dose, P<0.05): Control: 0.517±0.012g 2500 mg/kg: 0.573±0.008g 5000 mg/kg: 0.615±0.021g	control litters (P<0.001), amongst others cleft palate, cleft lip, anophthalmia, meningoencephalocele, gastroschisis, exencephaly. -Visceral malformations in 6 litters at low dose (P<0.05), 8 at high dose (P<0.01) compared to 0 control litters. Increase mainly due to anomalies of great vessels. - Skeletal malformations in 19 litters at mid dose and 24 litters at high dose versus 2 control litters (P<0.001), including malformed ribs (short, missing, branched and/or fused), malformed arches (enlarged, small, fused and/or missing) and malformed centra (misaligned, unilateral ossification, off center, fused and/or missing).	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rats						
				- Increased water consumption during and after treatment (mid and high dose, P<0.05).		
Price, 1988 [23]	COBS CD (SD)BR outbred albino rats N= 4-5 females per dose group N= 20 F1 litters per/dose group	Developmental study (NTP study, GLP compliant). <u>Design:</u> Treatment of pregnant rats from GD6-20 Dams were euthanised on PND1. Pups were euthanised PND 4, 22 or 63. <u>Examinations:</u> maternal organ weights, uterine contents, offspring organ weights, and visceral, skeletal and morphological abnormalities. <u>Statistics:</u> - Data are presented as mean ± SEM	Purity: 99.6% Vehicle: water <u>Concentrations:</u> 0, 250,1250 and 2250 mg/kg bw/day in water via oral gavage. Dose formulations were analysed by gas chromatography and compared to an internal standard to verify concentration prior to administration to the test animals.	<u>Maternal toxicity:</u> - No effect on body weight GD0-15, reduced body weight in high dose group on GD20 (P<0.01, 352.0±4.8 g compared to 367.8±4.3 g in control group). – Reduced gestational weight gain (P<0.01) in high dose group (84.3±3.5 g compared to 106.2±3.0 g in control group). - Increased kidney weight (P<0.01) in high dose group (2.42±0.07 g compared to 2.20±0.05 g in control group). - No significant effect on liver weight observed	<u>P0 parents</u> -Longer gestational periods (P<0.01) in high dose groups. Control: 21.26±0.07 days 1250 mg/kg: 21.58±0.07 days 2250 mg/kg: 31.84±0.09 days -Reduced live litter size (P<0.01) in high dose group (11.87±0.48 compared to 13.67±0.33 in control group). -Increased mortality per litter (P<0.01) in high dose group (5.15±1.06% compared to 2.07±1.12% in control group) <u>F1 offspring:</u> -Reduced body weight in high dose group on PND1 (P<0.01) and PND22 (P<0.05). -Reduced kidney weight in high dose group (P<0.05) on PND22 and PND63.	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rats						
		- General Linear Model analysis - one-way ANOVA and pairwise comparisons - Chi-square test (nominal data). One tailed- fisher exact test for pairwise comparison.		- Reduced uterine weight (P<0.05) in high dose group (2.08±0.14 g compared to 4.62±0.14 g in control group).	-Reduced brain weight in high dose group (P<0.05) on PND22 and in females on PND63. -Reduced time to achieve wire grasping in high dose group (P<0.05) (9.71±0.18 days compared to 11.98±0.78 days in control group). - No significant effect on external and visceral defects observed. - Increase in skeletal defects (P<0.001), litters with malformed pups in high dose group (female 60.0% and male 30% compared to 0% in control group), including malformed ribs (missing, short, fused), malformed centra (fused, ossification, off center) and fused sternebrae.	
Marr, 1992 [24]	Rat, Sprague-Dawley, N= 7/dose group/time point of euthanasia	Developmental study (non-guideline, non-GLP). <u>Design:</u> Treatment of pregnant rats from GD 6-15.	Purity>99% <u>Concentrations:</u> 0, 2500 mg/kg bw/day in water via oral gavage.	Maternal weights were decreased by 3-6% compared to control at GD 11, 15, 18 and 20 (all P<0.05).	**-** Foetal body weight was 24-26% less than control at GD 18 and 20 (P<0.05 for both time points). - Pup body weight was 10% less then control at PND 1 (P<0.05). <u>Skeletal malformations:</u>	When the ossification data were covaried by pup weight, there were less

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rats						
		Dams were euthanized on GD 18, 20 or PND 1, 4, 14, 21 or 63. <u>Examinations:</u> - Foetus/pup weight - Examination of skeleton Statistics: - Results are presented as mean ± SEM - Continuous data: t-test or Mann-Whitney U test. Categorical data: Fisher's Exact test - Proportions of ossification: general linear model (ANOVA) (with and without body weight as covariate)			- Increased percentage malformed per litter, exposed versus control: GD18: 76.3±11.3% versus 0%, P<0.05 GD 20: 88.4±3.4% versus 1.1±1.1%, P<0.05 PND 1: 95.2±4.8% versus 0%, P<0.05 PND 4: 83.4±6.1% versus 1.0±0.5%, P<0.05 PND 14: 77.5±16.5% versus 0%, P<0.05 PND 21: 87.5±7.2% versus 0%, P<0.05 PND 63: 28.2±8.3% versus 7.5±7.5%, not significant - Percentage of litters with malformations was 100% at GD18, GD 20, PND1, PND 21 (all P<0.05 compared to control) and 100% at PND 4 and 80% at PND 63 (not significant). - Agenesis of the ribs (incidence 24-41%) and short ribs (incidence 6-34%) were common.	statistically significant results.

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rats						
					- Bipartite cartilage and bipartite ossification centres, incidence peaks at PND 1 and 21. - Increase in variations in exposed group. Rudimentary rib on lumbar arch I was most common. <u>Ossifications (ANOVA without foetal body weight as covariate):</u> <u>GD20:</u> - Percent total ossification lowered (24.85±1.46% versus 37.42±1.19%, P<0.05) - Percent sternebrae, centra, forelimb distal phalanges and metarsals ossified were lowered (P<0.05). <u>PND1:</u> - Percent total ossification lowered (66.49±1.33% versus 78.64±0.45%, P<0.05). - Percent sternebrae and centra ossified lowered (P<0.05). <u>PND 4:</u> - Percent total ossification lowered (84.32±0.95% versus 92.58±0.98%, P<0.05).	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rats						
					- Percent sternebrae, centra, forelimb middle phalanges ossified were lowered (P<0.05). <u>PND 14:</u> - Percent total ossification lowered (94.08±0.54% versus 99.75±0.13%, P<0.05) - Percent sternebrae and centra ossified lowered (P<0.05). <u>PND21:</u> - Percent total ossification lowered (95.22±0.50 versus 100±0.0%, P<0.05). - Percent sternebrae and centra ossified lowered (P<0.05). <u>PND 63:</u> - Percent sternebrae ossified lowered (P<0.05)	
Neeper-Bradley, 1995 [25]	Rat, CD, females N= 25/group	Developmental study (EPA TSCA testing guideline, GLP compliant). <u>Design:</u> Females were treated daily from GD 5 through 15. Dams were euthanized on GD 21.	Purity: >99.9% Vehicle: water <u>Concentrations:</u> 0 (vehicle), 150, 500, 1000 and 2500 mg/kg bw/day, via oral gavage.	Maternal toxicity: <u>1000 mg/kg bw/day:</u> - Increased relative liver weight (P<0.05). <u>2500 mg/kg bw/day:</u> - Body weight gain was reduced at GD6-9 (P<0.05) and GD15-18 (P<0.01).	Developmental effects: <u>500 mg/kg bw/day:</u> Skeletal variations: - Increased incidence in poorly ossified supraoccipital, P<0.05 <u>1000 mg/kg bw/day:</u> - Reduced fetal body weight per litter (4.981±0.31 g versus	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rats						
		Examinations: - Maternal liver, kidney and gravid uterine weight. Kidney histology. - Corpora lutea, number of live and dead foetuses, resorptions. - Sex of foetuses, variations and malformations. Statistics: - Data presented as mean ±SD - Continuous data: ANOVA and t-tests with Bonferonni post-hoc test. - Non-parametric data: Kruskal-Wallis followed by Mann Withney U when appropriate. - Incidence data: Fisher exact test	Dosing solutions were 103-108% of nominal concentrations	- During treatment, water consumption was increased (P<0.01). - Reduced final body weight (365.08±33.0g versus 382.17±30.6g in controls), P<0.01. -Reduced gravid uterine weight (74.042±24.0g versus 98.013±30.6g in controls), P<0.01. - Increased absolute and relative kidney weight and relative liver weight (all P<0.01).	5.245±0.26 g in controls), P<0.05. Malformations external/soft tissue: - Gastroschisis in one foetus, not significant. Malformations skeletal: - Extra 14 thoracic centrum and arches (8/23 versus 1/24 control litter), P<0.05. - Missing rib (8/23 versus 0/24 control litters), P<0.01. - Missing thoracic arches (6/23 versus 0/24 control litters), P<0.05. Variations skeletal: Increased incidence of multiple skeletal variations involving ossification sites at the cervical and thoracic region. 2500 mg/kg bw/day: - Reduced foetal body weight per litter (4.033±0.40g versus 5.245±0.26g in controls), P<0.01.	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rats						
					Malformations external/soft tissue: - Gastroschisis (7/21 versus 0/24 litters in controls), P<0.05. - Hydrocephaly (10/21 versus 0/24 control litters), P<0.05. - Lateral ventricle dilated (6/21 versus 0/24 control litters), P<0.05. - Umbilical hernia (5/21 versus 0/24 control litters), P<0.05. - Atelectasis (19/21 versus 12/24 control litters), P<0.01. Skeletal malformations: - Cervical arch 7 missing (6/21 versus 0/24 control litters), P<0.05. - Extra 14 thoracic centrum and arches (18/21 versus 1/24 control litter), P<0.01. - Lumbar centra skewed (11/21 versus 0/24 control litters), P<0.05. - Rib missing (20/21 versus 0/24 control litters), P<0.01. - Ribs fused (17/21 versus 0/24 control litters), P<0.01.	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rats						
					- Thoracic arch missing (17/21 versus 0/24 control litters), P<0.01. - Thoracic arches fused (13/21 versus 0/24 control litters), P<0.01. - Thoracic centrum and arch missing (5/21 versus 0/24 control litters), P<0.05. <u>Variations skeletal:</u> Increased incidence of multiple skeletal variations involving ossification sites at the cervical and thoracic region.	
DePass, 1986 [16]	Fisher 344 Rats N= 10 males/ group and 20 females/ group	Three-generation reproduction study (non-guideline, non-GLP). <u>Design:</u> - During 7-week premating period - During breeding, time period not mentioned. - Offspring was exposed throughout life and started breeding on 100 days of age.	Purity: 99.93% <u>Concentrations:</u> Approximately 0.04, 0.2 and 1.0 g/kg bw/day in diet as calculated by the authors. Two untreated control groups.	No effects on mortality, diet consumption or body weight observed.	No treatment related developmental toxicity observed. Dominant lethal mutations observed in positive control but not ethylene glycol exposure groups.	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rats						
		Examinations: Fertility, behaviour and histopathology, kidney lesions. Statistics: continuous data using Barlett's test. T-test for equal and unequal variance. Frequency data using chi-square and Fisher's exact tests.				
Reference	**Species**	**Experimental period and design**	**Dose and route**	**General toxicity**	**Effects on reproductive organs or reproduction**	**Remarks**
Developmental toxicity studies in mice						
Schuler, 1984 [26]	Mouse, CD-1, females N=50/group	Developmental study (non-guideline, non-GLP) with a preparatory dose finding study. Design: Treatment of pregnant dams from GD 7 to 14. Pups were sacrificed 3 days after birth.	Purity: >99% Concentration: 11090 mg/kg bw/day, by oral gavage Vehicle: water Negative control: vehicle	Maternal mortality: - 5/50 versus 0/50 in controls (not significant)	- Decreased viable litters (litter with at least one viable pup): 15/37 versus 29/29 in controls (P<0.05) - Decreased number of live pups per litter: 2 versus 9 in controls (P<0.05). - Increased number of dead pups per litter: 1.5 versus 0.1 in controls (P<0.05). - Decreased pup postnatal survival: 40% versus 100% in controls (P<0.05).	Dose levels for range finding were not further specified. It is unclear if statistics were performed on maternal mortality.

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental toxicity studies in mice						
		Examinations: number of live pups, pup weight, maternal body weight. Statistics: - Body weight: analysis of variance - Mice with viable litters: Fisher-Irwin - Number of live pups and survival: student's t-test			- Decreased pup weight gain: 0.2g versus 7g in controls (P<0.05). - Decreased pup birth weight: 1.4g versus 1.7g in controls (P<0.05).	
Price, 1985 [22]	Mouse, CD-1, females N=10/group /replicate (so total of 20/group)	Developmental study (NTP study, GLP compliant). Design: Treatment of pregnant mice from GD6 through 15. Two replicates of the teratology evaluation were conducted. Caesarean section was performed on GD 17. Examinations: maternal organ weights, uterine contents and visceral,	Purity: >99% Vehicle: water Concentrations: 0, 750 (low), 1500 (mid) or 3000 (high) mg/kg bw/day, via oral gavage Negative control: vehicle Actual dose levels were within 10% of the calculated levels.	- Decreased weight gain during treatment in mid and high dose (P<0.05 for both groups). - Decreased maternal liver weight in mid and high dose groups(P<0.05 for both groups). - Decreased gravid uterine weight in mid and high dose (P<0.05 for both groups).	Developmental toxicity: - Reduction in the number of implantation sites per litter in the mid dose (11.57±0.70) compared to control (13.32±0.44), P<0.05. - Dose related increase in post-implantation loss per litter or at one or more site, but no significant pairwise comparisons. - Dose related decrease in live litter size, with significant decrease in the high dose group (9.83±0.56) compared to control (11.88 ±0.49), P<0.05.	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental toxicity studies in mice						
		skeletal and morphological abnormalities. <u>Statistics:</u> - Data are presented as mean ± SEM - Dose response: test for linear trend - ANOVA with post hoc tests - test for linear trend on proportions and chi-square test (nominal data). One tailed- fisher exact test for pairwise comparison.			- Dose related decrease in average foetal body weight per litter. Low dose (0.882±0.017g), mid dose (0.787±0.024g) and high dose (0.712±0.022g) were decreased compared to control (0.974±0.013g), P<0.01 for all groups. - Dose related increase in the percentage of malformed foetuses per litter. Low dose (10.0±1.96%), mid dose (37.77±6.30%) and high dose (56.54±6.80%) compared to control (0.25±0.25%), P<0.01 for all groups. - Dose related increase in the percentage of litters or with one or more malformed foetus. Low dose (66.67%), mid dose (81.82%) and high dose (96.65%) compared to control (4.0%), P<0.001 for all groups. <u>Malformations:</u> - External malformations in 8 litters (high dose) versus 0 in the controls, P<0.05, including exencephaly,	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental toxicity studies in mice						
					meningoencephalocele, cleft palate, cleft lip, facial cleft. - Dose response related increase in visceral malformations. Significant in high dose (7 litters) versus controls (0 litters), P<0.05. Visceral malformations are among others aortic stenosis, hydronephrosis, hydoureter, retrotracheal or retrooesophageal pulmonary artery. - Dose response related increase in skeletal malformations. Significant in low dose (15 litters), mid dose (17 litters) and high dose (22 litters) versus control (1 litter), P<0.01 for all groups. Malformations include malformed ribs (short, missing, branched and/or fused), malformed arches (enlarged, small, fused and/or missing) and malformed centra (misaligned, unilateral ossification, off centre, fused and/or missing).	
Neeper-Bradley, 1995 [25]	Mice, CD-1, females N=30/group	Developmental study (non-guideline, GLP compliant)	Purity: >99.9% Vehicle: water <u>Concentrations:</u>	<u>Maternal toxicity:</u> One death occurred in control group, 2 deaths/group occurred at	- Foetal body weights/litter were reduced at 1500 mg/kg bw/day (1.156±0.11 g versus	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental toxicity studies in mice						
		Design: Females were treated daily from GD5 through GD15. Dams were euthanized on GD18. Examinations: - Maternal liver, kidney and gravid uterine weight. Kidney histology. - Corpora lutea, number of live and dead foetuses, resorptions. - Sex of foetuses, variations and malformations. Statistics: - Data presented as mean ±Sd - Continuous data: ANOVA and t-tests with Bonferonni post-hoc test. - Non-parametric data: Kruskal-Wallis followed by Mann Withney U when appropriate. - Incidence data: Fisher exact test	0 (vehicle), 50, 150 (low), 500(mid) and 1500 (high) mg/kg bw/day, via oral gavage. Dosing solutions were 92-107% of nominal concentrations.	50, 500 and 1500 mg/kg bw/day (not significant). Slight reductions in weight gain and body weight at 1500 mg/kg bw/day (not significant).	1.325±0.09 g in controls), P<0.01. Malformations external/soft tissue: - Exencephaly noted in 2 litters (500 mg/kg bw/day) and 3 litters (1500 mg/kg bw/day), not significant. Skeletal malformations (500 mg/kg bw/day): - Extra rib 14, first lumbar arch, bilateral (17/24 versus 4/19 control litters), P<0.05. Skeletal malformations (1500 mg/kg bw/day): - 2-12 thoracic arches fused (8/21 versus 0/19 control litters), P<0.01. - 2-12 ribs fused (15/21 versus 1/19 control litters), P<0.01. - Extra 14 thoracic centrum and arches (10/21 versus 0/19 control litters), P<0.01. - Extra rib 14, thoracic arch 14: bilateral (10/21 versus 0/19), P<0.01.	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental toxicity studies in mice						
					Skeletal variations: Increased incidence of multiple skeletal variations involving ossification sites at cervical, thoracic and lumbar region as well in the head.	
Tyl, 1995c [27]	Mice, CD1, females N=30/group	Developmental dermal study (non-guideline, GLP compliant). Design: Dermal study with positive control gavage group. Females exposed daily on GD6 through 15. On GD18 mice were asphyxiated. Examinations: - Maternal organ weights, corpora lutea, kidney microscopy status of implantation sites. - Visceral and skeletal malformations and variations.	Purity: ~100% Vehicle: water Concentrations: 3000 mg/kg bw/day, via oral gavage. Negative control: 0% dermal application for 6h per day.	- 8 females died. - Gestational weight change increased (5.283±3.172g) versus controls (3.310±1.796g), P<0.05. Clinical signs: hypoactivity, cold extremities, hunched posture, urogenital discharge, and tissue on paperboard (from abortions). Increased incidence in kidney lesions: - tubular nephrosis (10/30) versus controls (0/30), P<0.01. - tubular cell degeneration (7/30)	- Reduced foetal body weight per litter (0.990±0.207g) versus controls (1.241±0.110g), P<0.001. -Increased number of litters with soft tissue malformations (11/16) versus control (7/23), P<0.05. - Increased number of litters with skeletal malformations (16/16) versus control (12/23), P<0.01. Visceral malformations: - Dilation of lateral ventricle (9/16 litters) versus controls (1/23 litters), P<0.01. Skeletal malformations: - Fusion of 2-12 thoracic arches	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental toxicity studies in mice						
		<u>Statistics:</u> - Data presented as mean ± SD - Continuous data: ANOVA and t-tests with Bonferonni post-hoc. - Nonparametric data: Kruskal-Wallis and Mann-Withney U. - Incidence data: Fisher-exact test.		versus control (0/30), P<0.05. - Autolysis (6/30) versus control (0/30).	- Fusion of lumbar arch (5/16 litters) versus controls (0/23 litters), P<0.05. - Fusion of lumbar centra (5/16 litters) versus controls (0/23 litters), P<0.05. - Fusion of sacral centra (4/16 litters) versus controls (0/23 litters), P<0.05. - Malaligned thoracic centra (7/16 litters) versus controls (0/23 litters), P<0.01 - Missing thoracic arches (4/16 litters) versus controls (0/23 litters), P<0.05. - Short rib (4/16 litters) versus controls (0/23 litters), P<0.05. - Fusion of 2-12 ribs (15/16 litters) versus controls (1/23 litters), P<0.01. Variations: - Incidence significantly increased of 56 skeletal variations, mostly poorly/unossified bones, fused sternebrae, enlarged sagittal suture and skewed thoracic centra.	
Lamb, 1985 [14]	COBS Crl:CD1,	Reproductive study (non-guideline, non-GLP).	Purity: 99.6%	No general toxicity, i.e. clinical signs, effects on	<u>Offspring defects:</u>	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental toxicity studies in mice						
	(ICR)BR outbred albino mice (CD1 mice) N=20/sex/group Controls: 40/sex	Fertility assessment by continuous breeding with a preparatory dose range finding study. <u>Design:</u> - Premating period of 7 weeks - 98 days cohabitation and breeding (male+female), all pups born during this period were killed - 21 days segregation period, pups born during this period were kept alive - Exposure occurred during premating, cohabitation, segregation and throughout the life of offspring. - High dose and control pups (N=20/sex/group) born during segregation were mated. <u>Examinations:</u>	<u>Concentrations:</u> 0, 0.25, 0.5 and 1.0% (weight/volume), oral via drinking water. According to the authors, this was equal to 410, 840 and 1640 mg/kg bw/day, based on daily water consumption per pair of mice for 2 weeks and mean body weight data. Dosage solutions were within 98 – 107% of intended concentrations.	body weight or water consumption in parental animals. Some deaths occurred in all groups. One death in the 0.5% group could be treatment related according to the authors (oxalate crystals in renal tubules).	- Live pup weight decreased at high dose (1.53±0.02 g) versus control (1.63±0.02 g), P<0.01. - A pattern of skeletal defects (affecting skull, sternebrae, ribs and vertebrae) in treated mice, not in controls (not quantified). Defects included shortened facial bones, fused ribs, abnormally shaped or missing sternebrae, abnormally shaped vertebrae and twisting of spine.	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental toxicity studies in mice						
		- Fertility and reproductive performance of the adults and F1 generation. Viability, sex and weight of the pups. - Organ weights of F1 offspring and skeletal examination of a proportion of F1 mice. - Histology of the head of F1 mice. <u>Statistics:</u> - Results presented as Mean ±SE. - Tests used differ per parameter. Mostly Chi-Square approximation to Kruskall Wallis is used for group comparisons and Mann-Withney U or Fisher exact test for pairwise comparisons. An ANOVA was carried out for assessment of body weight.				

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental toxicity studies in mice						
Gulati, 1986 [15]	Crl:CD-l (ICR)BR outbred albino mice (CD1) N=20/sex/group Controls: 40/sex	Continuous breeding study (non-guideline, GLP-compliant). <u>Design:</u> - Premating period of 7 weeks - 98 days cohabitation and breeding (male+female), all pups born during this period were killed - 21 days segregation period, pups born during this period were kept alive - Exposure occurred during premating, cohabitation, segregation and throughout the life of offspring. - Pups (N=20/sex/group) born during segregation were mated. <u>Examinations:</u> - Fertility, viability and weight of litters.	<u>Purity:</u> 99.6% <u>Concentrations:</u> 0, 0.5, 1, 1.5% (weight/volume), oral in drinking water. This is equivalent to 0, 897, 1798 and 2826 mg/kg bw/day, based on daily water consumption and mean body weight data, as calculated by the authors. Dosage solutions were within 94 and 104% of intended concentration.	<u>F0 Parental animals:</u> - No treatment-related effects on body, liver or kidney weight observed in female mice. - Significant reduction in body (42.29±0.10g in 1.5% group vs 46.45±1.04g in control group) and liver weight (1.93±0.05g in 1.5% group vs 2.11±0.07g in control group) observed in male mice. <u>F1 Parental animals:</u> - 1.5% ethylene glycol resulted in statistically significant (P<0.05) reduced liver weight in females (1.52±0.05g, vs 1.65±0.05g in control groups) and males (1.69±0.05g vs 1.8±0.05g).	<u>F0 offspring:</u> - Statistically significant (P<0.01) reduction in adjusted live pup weight was observed in 1.0% (1.53±0.02 g) and 1.5% (1.48±0.02 g) compared to control group (1.58±0.01g). <u>F1 offspring</u> - Statistically significant (P<0.05) reduction in adjusted live pup weight was observed in 0.5% (1.46±0.03g), 1% (1.46±0.03g), and 1.5% (1.45±0.03g) compared to control group (1.54±0.03g). <u>Offspring deformities:</u> - Increase in incidence of left/lip palate in 1% (N=17) and 1.5% (N=28) compared to control group (N=0). - Increase in incidence of ablepheron in 1% (N=19) and 1.5% (N=21) compared to control group (N=0).	The total number of offspring examined for deformities is unclear.

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental toxicity studies in mice						
		- Organ weight and skeletal deformities in offspring. <u>Statistics:</u> - Data is displayed as mean ±SE. - Cochran-Armitage or Jonckheere test for trends. Statistic differ per parameter, mostly used are Chi-Square, Kruskal-Wallis, Wilcoxon rank-sum and Fisher's exact test.				
Harris, 1992 [18]	Crl:CD-1 mice N=10/sex/group	Short-Term Reproductive and Developmental Toxicity Screening study (non-guideline, non-GLP). <u>Design:</u> Treatment of pregnant female mice during GD8-14. <u>Examinations:</u> - Liver, kidney and testes histology in male mice.	Purity not mentioned. <u>Concentrations:</u> 0, 250, 700, and 2500 mg/kg/day in water via oral gavage. Dosing solutions within 93-100% of target concentrations.	No adverse clinical signs observed. No mortality observed.	2500 mg/kg/day exposure during gestation resulted in a significant ($P<0.05$) decrease in total litter weight on PND1 (15.3±0.9 g vs 19±1.1 g in control group) and PND4 (26.6±1.5 g vs 31.7±1.3 g in control groups).	Fertility effects observed in this study are described in section 8.1.

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental toxicity studies in mice						
		- Fertility and litter weight. <u>Statistics:</u> Cochran-Armitage test for linear trend followed by Fisher's exact test. Kruskal-Wallis analysis of variance for dose group comparison and Jonckheere's test for dose-responses. Mann-Whitney U test for pairwise comparisons.				

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rabbits						
Tyl, 1993 [28]	Rabbits, New Zealand white, females, N=23-24/group	Developmental study (non-guideline, GLP compliant). <u>Design:</u> Treatment of pregnant rabbits from GD6 through 19. Pregnant rabbits were euthanized on GD 30.	<u>Purity:</u> 98% Vehicle: water Oral gavage <u>Concentrations:</u> 0, 100, 500, 1000 or 2000 mg/kg bw/day	<u>At 2000 mg/kg bw/day;</u> - 42.1% mortality - three early deliveries - one spontaneous abortion - absolute kidney weight slightly increased (106.3% right kidney; 107.6% of the control of the left kidney),	No indication of developmental toxicity at any dose tested.	NTP study

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Developmental studies in rabbits						
		Examinations: - Maternal liver, kidney and intact uterine weight. Kidney histology. - Number of corpora lutea, implantations, live and dead foetuses, and resorptions counted. - Foetus weight, foetus abnormalities, variations and malformations. Statistics: - General Linear Trend Models procedures applied for ANOVA of maternal and foetal parameters. - Prior to GLM analysis, an arcsine-square root transformation was performed on all litter-derived percentage data - Bartlett's test for homogeneity of variance was performed on all data to be analysed by ANOVA.		accompanied with renal lesions limited to the cortical renal tubules The death of the pregnant females at 2000 mg/kg bw/day was directly related to the acute renal failure.		

8.2.1 Developmental toxicity studies in rats (oral exposure)

Maronpot, 1983

A non-guideline prenatal development study was performed with Fischer 344 rats [21]. Ethylene glycol (purity >99.9%) was administered to pregnant dams via the diet at target doses of 0, 40, 200, and 1000 mg/kg bw/day. Dams were treated from GD6 to 12 and a c-section was performed at GD21. General toxicity in the dams was not observed. At a target dose of 1000 mg/kg bw/day, preimplantation loss was higher compared to control but not statistically significant. Also, increased incidences of poorly ossified and unossified vertebral centra were observed at this dose. Major malformations occurred only in the positive controls.

Price, 1985

A non-guideline developmental toxicity study was performed by Price et al. in 1985 [22]. The study was GLP compliant and performed by the NTP. Female CD rats (n=10/group) were treated with ethylene glycol (purity: 99%) at 0 (vehicle), 1250, 2500 or 5000 mg/kg/day via oral gavage from GD6 through 15. C-section was performed on GD20. Two replicates of the study were performed making the total number of animals per group 20 instead of 10.
All treated rats showed piloerection and treated rats at the mid and high dose showed an increased water consumption compared to control. There was a dose related decrease in all maternal body weight parameters and gravid uterine weight. Most effects were statistically significant at the mid and high dose only, but weight gain during treatment was significantly lowered in all treatment groups. Maternal liver weight (high dose) and relative kidney weight (mid and high dose) were significantly decreased compared to control.
The following developmental toxicity parameters were increased or decreased in a dose-related manner: an increase in post-implantation loss per litter or at one or more site was observed, as well an increase in the percentage of malformed live foetuses per litter and the percentage litters with one or more malformed live foetuses. There was decrease in the number of lie foetuses per litter and the average foetal body weight. Post-implantation loss was significantly increased at the high dose only. The number of live foetuses per litter as well as the average foetal body weight per litter were significantly decreased at mid and high dose. There was also an increase in the percentage of live foetuses that were malformed as well as in the percentage of litters with at least one malformed foetus both at the mid and high dose. For all morphological defects, there was a dose response relationship observed. External and visceral malformations were significantly increased at the high dose and skeletal malformations and the mid and high dose. Malformations included, amongst others, cleft palate, cleft lip, anopthalmia, meningoencephalocele, gastroschisis, exencephaly, anomalies of great vessels and malformations of ribs, arches and centra.

Price, 1988

A non-guideline developmental study was performed by Price et al. (1988) (GLP compliant; performed by the NTP). Female CD rats (N=4-5/group) were treated with ethylene glycol (purity: 99.6%) at 0, 250, 1250 and 2250 mg/kg bw/day via oral gavage during GD6-20. A vehicle control group was included. Dams were euthanized on PND 1. A reduced

body weight, reduced gestational weight gain, increased kidney weight and reduced uterine weight was observed in the high dose group. Statistically significant longer gestational periods were observed in the 1250 and 2250 mg/kg bw/day exposure groups. A reduced live litter size and increased mortality per litter was observed in the high dose group.
Offspring showed reduced body weight on PND 1 and 22 in the high dose groups. A reduced kidney and brain weight was observed on PND 22 and 63 in the high dose groups. 2250 mg/kg bw/day exposure also resulted in a reduced time to achieve wire grasping. Ethylene glycol exposure did not result in external or visceral defects but did show an increase in skeletal malformations in the high dose group. 30% of the males and 60% of the females in this group showed malformations, compared to 0% in control groups. These malformations include malformed ribs, centra and sternebrae.

Marr, 1992
A non-guideline developmental study was performed by Marr et al. in 1992 [24]. Pregnant Sprague-Dawley rats were treated with ethylene glycol in water (2500 mg/kg/day, purity >99%) via oral gavage from GD 6-15. A vehicle control group was included. Dams were euthanized on GD 18, 20 or PND 1, 4, 14, 21 or 63. The number of treated dams were 7 per group per time point of euthanasia. The focus of the study was on skeletal malformations and ossifications.
Maternal weights were statistically significantly decreased by 3-6% compared to control at GD 11, 15, 18 and 20. Foetal body weight was approximately 25% less than controls at GD 18 and 20 (both $P<0.05$) and pup weight was 10% less than controls at PND 1 ($P<0.05$). Skeletal malformations occurred in 100% of the exposed litters at GD 18, 20 and PND1, 4 and 21. Also the percentage of malformed pups or foetuses per litter was 75% or higher in exposed groups versus less than 1% per litter in control groups (all statistically significant), except for PND 63 where only 28% was malformed. Agenesis of the ribs and short ribs were common malformations. According to the authors, skeletal remodelling in postnatal period may explain decrease in malformations on PND 63.
There was also an increase in variations in the exposed group. The percentage of total ossifications were statistically significantly lowered at all time points except PND 63 and could in part be attributed to lower ossifications of sternebrae and centra. The percentage of ossifications was mostly 5-10% lower than controls. Of note is that when the ossification data were covaried by pup weight, there were less statistically significant results which is why the authors propose that effects on ossification are mediated through foetal growth.

Neeper-Bradley, 1995
A non-guideline developmental study was performed on female CD rats (n=15/group) [26]. They were treated once daily with ethylene glycol from GD5 through GD 15 at dose levels of 0 (vehicle), 150, 500, 1000 and 2500 mg/kg bw/day via oral gavage. Dams were euthanized on GD21. Maternal toxicity was observed mostly at 2500 mg/kg bw/day. Body weight gain was significantly reduced at GD6-9 and GD15-18 and the final body weight was also reduced compared to control. During treatment, water consumption was significantly increased. Gravid

uterine weight was decreased compared to controls and absolute and relative kidney weights as well as relative liver weight were significantly increased. At 1000 mg/kg bw/day, relative liver weight was also significantly increased.
Most malformations also occurred at a dose of 2500 mg/kg bw/day. A number of external or soft tissue malformations were observed such as gastroschisis, hydrocephaly, severe lateral ventricle dilation, umbilical hernia and foetal atelectasis. Increased incidences of 31 skeletal malformations were observed primarily in the thoracic region, for example missing ribs or thoracic arches (full details in overview table). At 500 mg/kg bw/day an increased incidence in poorly ossified supraoccipital was observed. At 1000 mg/kg bw/day, there was one foetus with gastroschisis (not significant) and increased incidences in a number different types of skeletal malformations were observed.

DePass, 1986
A non-guideline three generation reproduction study was performed by DePass et al. in 1986 [16]. Fisher 344 Rats (n=10 males and 20 females/group) were treated with 0.04, 0.2, and 1.0 g/kg/day ethylene glycol (purity >99.9%) in diet. Rats were exposed during a 7-week premating period and throughout breeding. Offspring (n=10 males and 20 females/group randomly chosen from the litters) were exposed throughout life and breeding was started at 100 days of age. No treatment related effects on mortality, diet or body weight changes were observed. No treatment related effects on fertility or histopathology were observed. One second generation female and one second generation male in the high dose group showed mild focal interstitial nephritis but this condition was also observed in two control group pups. One third generation female in the high dose group showed mild focal tubular hyperplasia but this condition was also observed in two control male pups.

8.2.2 Developmental toxicity studies in mice (oral exposure)

Schuler, 1984
A non-guideline developmental study was performed with female CD-1 mice [22]. First, a dose range-finding study was performed to determine the LD50. For the developmental study, pregnant dams (n=49) were treated with 1400 mg/kg bw/day ethylene glycol (purity: 99%) via oral gavage from GD7 to GD14. The selected dose was the LD10. A vehicle control group was included (n=50). Pups were sacrificed three days after birth. Mortality in treated pregnant dams was higher than in controls, but it is unclear whether statistics were performed for this parameter. The number of viable litters was decreased in treated dams compared to control and the number of dead pups per litter was increased. The number of live pups per litter, the pup postnatal survival, pup weight gain and pup birth weight were all significantly decreased compared to controls.

Price, 1985
A non-guideline developmental toxicity study was performed by Price et al. [23]. The study was GLP compliant and performed by the NTP. Female CD-1 mice (n=10/group) were treated with ethylene glycol (purity: 99%) at 0 (vehicle), 750, 1500 or 3000 mg/kg/day via oral gavage from GD6 through 15. C-section was performed on GD17. Two

replicates of the study were performed making the total number of animals per group 20 instead of 10.
Dams of the mid and high dose group showed a significantly decreased weight gain during treatment, a decreased liver weight and decreased gravid uterine weights. The number of implantation sites per litter in the mid dose was significantly lowered. There was a dose related increase in post-implantation loss per litter or at one or more site, but there were no significant pairwise comparisons. Also, there was a dose related decrease in live litter size with a significant decrease in the high dose group as well as a dose-related decrease in average foetal body weight per litter where foetal body weights in all groups were significantly decreased. Regarding malformations, there was a dose related increase in the percentage of malformed foetuses per litter and in the percentage of litters with at least one malformed foetus, the percentages were significantly increased in all dose groups. External and visceral malformations were significantly increased in the high dose group. Skeletal malformations were significantly increased at all dose levels. Malformations included, amongst others, exencephaly, meningoencephalocele, cleft palate, cleft lip, facial cleft, aortic stenosis, hydronephrosis, hydoureter, retrotracheal or retroesophageal pulmonary artery and malformations of ribs, arches and centra.

Neeper-Bradley 1995
A non-guideline developmental study was performed on female CD-1 mice (n=30/group) [26]. They were treated once daily with ethylene glycol from GD5 through GD 15 at dose levels of 0 (vehicle), 50, 150, 500, and 1500 mg/kg bw/day via oral gavage. Dams were euthanized on GD18. Some deaths occurred in treatment groups 50, 500 and 1500 mg/kg bw day (not significant versus control). Slight reductions in maternal weight gain and body weights at 1500 mg/kg bw/day, but again not significant.
Foetal body weights/litter were reduced at 1500 mg/kg bw/day. Exencephaly was noted in 2 litters at 500 mg/kg bw/day and 3 litters at 1500 mg/kg bw/day, however this was not significant. One significant skeletal malformation (extra rib) occurred at 500 mg/kg bw/day. Many different types of skeletal malformations and variations occurred at 1500 mg/kg bw/day which were statistically significant. These occurred mostly in the axial/thoracic regions.

Tyl, 1995c
A non-guideline developmental dermal study was performed by Tyl et al. in 1995c [27]. As part of the dermal study, a positive control group with oral ethylene glycol was included of which the results are described here. The results of the dermal study are described in another section. Female CD-1 mice were treated daily with ethylene glycol (3000 mg/kg bw/day) from GD6 through 15 via oral gavage. The negative control comparison was 0% ethylene glycol applied dermally for 6h per day. On GD18 mice were euthanized.
Eight treated females died and multiple clinical signs were observed, such as hypoactivity or hunched posture. Gestational weight change significantly increased. There was an increased incidence in kidney lesions, namely tubular nephrosis, tubular cell degeneration and autolysis.

Foetal body weights per litter were significantly reduced. The number of litters with soft tissue malformations and/or skeletal malformations were significantly higher in treated mice. The increased visceral malformation was dilatation of lateral ventricles of the brain with tissue compression. The increased skeletal malformations were fusion of thoracic, lumbar and sacral arches and/or centra, misaligned thoracic centra, missing thoracic arches, short rib and fusion of 2-12 ribs. Additionally, incidences 55 skeletal variations occurred specifically in the treated group. Mostly incidences of variations were increased and statistically significant.

Lamb, 1985

A non-guideline fertility assessment by continuous breeding was performed by Lamb et al. in 1985 [14]. In this study, also some developmental effects were reported which are summarized below. CD-1 mice (20/sex/group) were dosed at 0.25, 0.5 and 1% ethylene glycol in drinking water. This is equal to 410, 840 and 1640 mg/kg bw/day, based on daily water consumption and mean body weight data. A control group of 40/sex was present. Exposure occurred during a premating period (7 weeks), a male/female cohabitation and breeding period (98 days), a segregation period (21 days) and throughout the life of the offspring.
No general toxicity occurred in the parental animals, but some deaths occurred in all groups. According to the authors, one death in the 0.5% group could be treatment-related as oxalate crystals in the renal tubules were found. At high dose, a decreased live pup weight and lower number of live pups per litter were observed. The fertility of the treated offspring was 20% lower compared to controls but this was not statistically significant. Also the number of live pups per litter and live pup weights were decreased (not significant). A pattern of skeletal defects was observed in the offspring in treated mice only, although this was not quantified. Skull, sternebrae, ribs and vertebrae were affected in both males and females.

Gulati, 1986

A non-guideline continuous breeding study was performed by Gulati et al. in 1986. CD-1 mice (20/sex/group) were dosed at 0.5, 1.0 or 1.5% ethylene glycol in drinking water, 0% controls were included (40/sex) [15]. This is equal to 0, 897, 1798 and 2826 mg/kg bw/day, based on daily water consumption and mean body weight data. Mice were exposed during the premating period (7 weeks), during the breeding period (98 days), the segregation period (21 days) and throughout the life of the offspring. Offspring in all dose groups were mated (20/sex/group). A significant reduction in body weight and liver weight was observed in parental male mice. No effects on body weight or treatment-related lesions in kidney, liver, ovary, uterus or vagina were observed in female mice. A significant reduction in adjusted live pup weight was observed in all dose groups compared to controls. In offspring, 1.5% ethylene glycol exposure showed a significant reduction in liver weight in both female and male mice, and a significant reduction in testes weight in male mice. A significant reduction in adjusted live pup weight was observed in all dose groups. Offspring pups showed increased incidences of facial deformities, such as cleft/lip palate and ablepharon, which were not observed in control groups.

Harris, 1992
A non-guideline 21-day developmental toxicity study was performed by Harris et al. in 1992 [19]. CD-1 mice (n=10/sex/group) were exposed to 0, 250, 700, and 2500 mg/kg/day ethylene glycol in drinking water. Females were treated between gestation days 8 and 14. No adverse clinical signs were observed, and the 2500 mg/kg/day ethylene glycol exposure group showed a significant decrease in total litter weight.

8.2.3 Developmental toxicity studies in rabbits (oral exposure)

Tyl et al., 1993
New Zealand White rabbits (N=23-24/dose) were administered ethylene glycol by gavage on gestation days 6-19 at doses of 0, 100, 500, 1000, and 2000 mg/kg bw/day [25]. After exposure to 2000 mg/kg bw/day, 42.1% mortality, three early deliveries and one spontaneous abortion was observed. Maternal absolute kidney weight (but not relative weight) was slightly increased at 2000 mg/kg bw/day (to 106.3% of the control value of the right kidney and 107.6% of the control of the left kidney). This was accompanied with renal lesions, which were limited to the cortical renal tubules and included intraluminal crystals, epithelial necrosis, and tubular dilation and degeneration. The death of the does at 2000 mg/kg bw/day was directly related to the acute renal failure from crystal deposition, consistent with third-stage toxicity, including the presence of oxalate crystals. No dose-related maternal toxicity occurred at ≤1000 mg/kg bw/day. There was no indication of developmental toxicity at any dose tested, including no effects on pre- or post-implantation loss, number of foetuses, foetal body weight, or sex ratio (% male foetuses) per litter, and no evidence of teratogenicity.

Table 6 Inhalation studies on ethylene glycol

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Tyl, 1995a [29]	COBS CD (SD)BR outbred albino rats N=25/sex/group	Developmental toxicity study (non-guideline, GLP compliant). Design: Whole-body exposure during GD days 6 to 15 for 6 hours per day. Examinations: body weight, organ weight, gestational parameters, developmental defects. Statistics: - Data displayed as mean ±SD. - Continuous variables using Levene's test for equal variances, ANOVA, and t-tests with Bonferroni probabilities. Non-parametric data using Kruskal-Wallis test followed by Mann-Witney U test. Incidence data using Fisher's exact test. ($p<0.05$ two tailed).	Test item: Respirable aerosol of ethylene glycol (2.3 µm) in inhalation chambers. Purity: >99.9% Target concentrations: 0, 150, 1000, 2500 mg/m^3 Analytical concentrations: 0, 119±13, 888±149, and 2090±244 mg/m^3	- Significant increase in liver weight in 2500 mg/m^3 exposure groups (15.001±1.31g vs 13.841±1.72g in control group) - No effects on food, water consumption, body weight, or weight gain observed.	-No effect on gestational parameters or malformations.	Ethylene glycol measured in fur indicates oral exposure, calculated to be the major route of exposure (64-90%).

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Tyl, 1995a [29]	CD-1 (Crl:CD-1 (1CR)BR) outbred albino mice N=25/sex/group	Developmental toxicity study (non-guideline, GLP compliant). Design: Whole-body exposure during GD days 6 to 15 for 6 hours per day. Examinations: Body weight, organ weight, gestational parameters, developmental defects. Statistics: - Data displayed as mean ±SD. - Continuous variables using Levene's test for equal variances, ANOVA, and t-tests with Bonferroni probabilities. Non-parametric data using Kruskal-Wallis test followed by Mann-Witney U test. Incidence data using Fisher's exact test. (p<0.05 two tailed).	Test item: Respirable aerosol of ethylene glycol (2.3 µm) in inhalation chambers. Purity: >99.9% Target concentrations: 0, 150, 1000, 2500 mg/m^3 Analytical concentrations: 0, 119±13, 888±149, and 2090±244 mg/m^3	-Significant reduction in maternal body weight in 1000 mg/m^3 (49.841±5.13g, P<0.05) and 2500 mg/m^3 (47.391±5.46g P<0.001) groups compared to control groups (53.211 g±3.33) -Wet fur observed in all ethylene glycol exposure groups.	-Reduced gravid uterine weight in 1000 mg/m^3 and 2500 mg/m^3 groups (both P<0.001) -Reduction in viable implants in 2500 mg/m^3 group (8.0±2.9 vs 10.7±1.8 in control group, P<0.001) - Increase in non-viable implants per litter in at 1000 mg/m^3 (2.9±2.0, P<0.01) and 2500 mg/m^3 (4.2±2.9, P<0.001) compared to control groups (1.4±1.0) - Decreased percentage live foetuses in 1000 mg/m^3 (P<0.01) and 2500 mg/m^3 (P<0.001) compared to control groups - Change in sex ratio in 1000 mg/m^3 group compared to control group (P<0.01) - Reduction (P<0.001) in foetal body weight in 1000 (1.07g± 0.14) and 2500 (0.94±0.14g) mg/m^3 exposure groups compared to control groups (1.33±0.08g). - Increase (P<0.05) in incidence of external malformations in 1000	Ethylene glycol measured in fur indicates oral exposure, calculated to be the major route of exposure (64-90%).

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
					(30.4%) and 2500 (72.7%) mg/m^3 groups compared to control group (4%). - Increase (P<0.05) in incidence of visceral malformations in 1000 (34.8%) and 2500 (72.7%) mg/m^3 groups compared to control group (%). - Increase (P<0.05) in incidence of skeletal malformations in 1000 (100%) and 2500 (100%) mg/m^3 groups compared to control group (72%).	
Tyl, 1995b [30]	CD-1 (Crl:CD-1 (1CR)BR) outbred albino mice N=30/sex/group	Developmental toxicity study (non-guideline, GLP compliant). <u>Design:</u> Whole-body or nose-only exposure on GD days 6 to 15 for 6 hours per day <u>Examinations:</u> clinical signs, water consumption, uterus, liver and kidney weight, foetus examination. <u>Statistics:</u>	<u>Test item:</u> Ethylene glycol (1.7 µm). <u>Nose-only:</u> - Target concentration: 0, 500, 1000 and 2500 mg/m^3 - Analytical concentration: 0, 360, 779 and 2505 mg/m^3 <u>Whole body:</u> (positive control):	<u>Nose only:</u> - Body weight, weight gain and liver weight unaffected by treatment. -Increased kidney weight at 1000 (0.46± 0.046 g, P<0.05) and 2500 (0.472±0.034 g, P<0.01) mg/m^3 compared to control group (0.431±0.040g) - Wet fur in nose-only group (around the head) at 2500 mg/m^3	<u>Nose only:</u> - Non-significant and slight reduction of live foetuses per litter in 1000 and 2500 mg/m^3 groups. - Reduction in foetal body weight observed in 2500 mg/m^3 group (1.126±0.107g vs 1.289±0.126g in control group, P<0.001) - No increase in external or visceral malformations observed. - Increase in litters with at least one foetus with fused ribs in 2500 mg/m^3 group	

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
		- Data displayed as mean ±SD. - Continuous variables using Levene's test for equal variances, ANOVA, and t-tests with Bonferroni probabilities. Non-parametric data using Kruskal-Wallis test followed by Mann-Witney U test. Incidence data using Fisher's exact test. ($p<0.05$, two tailed).	- Target concentration: 0, 2100 mg/m^3 - Analytical concentration: 0, 2008 mg/m^3		(8/21 vs 1/22 in control group, $P<0.05$). <u>Whole body</u> (positive control): - Reduced gravid uterine weight (17.96±3.99 vs 20.70±4.49 in control group, $P<0.05$). - Increase in non-viable implants per litter ($P<0.01$) and reduced percentage of live foetuses per litter ($P<0.01$). - Reduction in foetal body weight (1.13±0.11 vs 1.36±0.13 in control group, $P<0.001$). - Increase in skeletal malformations (96.3% vs 10.3 in control group, $P<0.01$), including fused arches and ribs.	

Tyl, 1995a
A non-guideline developmental inhalation study was performed by Tyl et al. in 1995 [29]. Female CD rats and CD-1 mice were exposed to ethylene glycol for 6 hours per day from GD6 through GD15. Ethylene glycol was nebulized, and animals were exposed to 0, 150, 1000, 2500 mg/m^3 ethylene glycol aerosols (2.4 µm) in inhalation chambers. The purity of ethylene glycol was reported to be close to 100%. No mortality was observed within this study. No effects on food or water consumption, body weight or weight gain observed. The highest dose exposure group rats showed an increase in liver weight. No effects on gestational parameters or malformations observed within rats. CD-1 mice showed a significant reduction in maternal body weight and uterus weight in the 1000 and 2500 mg/m^3 exposure groups. Reduction in viable implants in 2500 mg/m^3 exposure group. A significant increase in the incidence of a number of external, visceral and skeletal malformations was observed in the 1000 and 2500 mg/m^3 exposure group, such as cleft palate, exencephaly, misshapen nasopharynx, and protruding tongue. Wet fur was observed in all ethylene glycol exposure groups and oral exposure is calculated to be the major route of exposure, the extent of which depending on the assumed retention of the chemical (64-90%).

Tyl, 1995b
A non-guideline developmental inhalation study was performed by Tyl et al. in 1995b [30]. Female CD-1 mice were exposed to ethylene glycol for 6 hours per day from GD6 through GD15. Ethylene glycol was nebulized, and animals were exposed to 0, 150, 1000, 2500 mg/m^3 ethylene glycol aerosols (1.7 µm) through nose-only or to 0 or 2100 mg/m^3 ethylene glycol aerosols through whole body exposure. The purity of ethylene glycol was reported to be close to 100%. Mortality was observed in control and 2500 mg/m^3 ethylene glycol nose-only exposure groups, found to be due to asphyxiation related to exposure method, not treatment chemical. The whole-body exposure group showed similar effects as described in Tyl et al. 1994. Nose-only ethylene glycol exposure did not result in alteration of body weight, weight gain or liver weight due to treatment. Increased kidney weight was observed in the 1000 and 2500 mg/m^3 exposure groups. A non-significant reduction in live foetuses per litter was observed in the 1000 and 2500 mg/m^3 exposure groups and a significant reduction in foetal body weight was observed in the 2500 mg/m^3 exposure group only. No treatment-related increases in external or visceral malformations were observed. A significant increase in the incidence of fused ribs was observed in the 2500 mg/m^3 exposure group

Table 7 Dermal studies on ethylene glycol

Reference	Species	Experimental period and design	Dose and route	General toxicity	Effects on reproductive organs or reproduction	Remarks
Tyl, 1995c [27]	Mice, CD1, females	Non-guideline developmental dermal study. Females exposed daily (6h occlusion of skin and restraining) on GD6 through 15. On GD18 mice were asphyxiated. Examinations: - Maternal organ weights, corpora lutea, kidney microscopy status of implantation sites. - Visceral and skeletal malformations and variations. Statistics: - Continuous data: ANOVA and t-tests with Bonferonni post-hoc. - Nonparametric data: Kruskal-Wallis and Mann-Withney U. - Incidence data: Fisher-exact test.	Purity: ~100% Vehicle: water Concentrations: 0% (vehicle), 12.5%, 50% and 100% (corresponds to 0, 404, 1677 and 3549 mg/kg bw/day) ethylene glycol, cutaneous in 0.1 mL/animal. Authors converse ethylene glycol dose by using the mean control body weights from GD6-15. Positive control: ethylene glycol 3000 mg/kg bw/day, via oral gavage. Actual dose levels were 99.2-101.2% of target levels.	- No deaths in treatment groups. - All clinical signs were associated with restrain conditions. - Gestational weight change increased at 100% (5.044±2.055g) versus controls (3.310±1.796g), P<0.05. - Minimal-grade tubular lesions in 3/30 mice in high dose group.	- Number of dams with fully resorbed litters was higher in all treatment groups compared to control. - Reduced number of late resorptions/litter at 12.5% (0.0±0.20) versus control (0.3±0.54), P<0.05. - Reduced number of dead foetuses/litter at 12.5% (0.1±0.27) versus controls (0.4±0.71), P<0.05. - No increased incidences in malformations. Variations: - Increased incidence in poorly ossified parietal skull bone at 100% - Increased incidence in majority of the intermediate phalanges of the hindlimb unossified at 100%.	Multiple signs of toxicity in the positive control group, results are specified in table on oral studies.

Tyl, 1995c

A non-guideline developmental dermal study was performed by Tyl et al. in 1995c [27]. Female CD-1 mice were exposed daily to ethylene glycol from GD 6 through 15. Ethylene glycol was dissolved in water and applied in concentrations of 0% (vehicle), 12.5%, 50% or 100% on the skin for 6h per day during which the skin was occluded and the mice were restrained. The concentrations correspond to 0, 404, 1677 and 3549 mg/kg bw/day. Oral ethylene glycol (3000 mg/kg bw/day) was used as a positive control. Mice were euthanised on GD 18.

There were no deaths in the treatment groups and all clinical signs during treatment were associated with the restrain conditions. Gestational weight change increased significantly in high dose mice. Minimal-grade tubular lesions were observed in 3/30 mice in the high dose group. The number of dams with fully resorbed litters was higher in all treatment groups compared to controls. There was a significantly reduced number of late resorptions per litter at 12.5% as well as a significantly reduced number of dead foetuses per litter. There were no increased incidences in malformations, but two variations occurred in the high dose group, namely a poorly ossified parietal skull bone and the majority of the intermediate phalanges of the hindlimb were unossified. Incidences of both variations were statistically significantly increased. Multiple signs of general and reproductive toxicity occurred in the positive control group which are described separately in the section on oral studies with ethylene glycol.

8.3 Adverse effects on or via lactation

No relevant information available.

8.4 Studies on cryopreservation and development of ovaries, sperm and embryos

The cryopreservation as well as the studies on development of ovaries, sperm and embryos after exposure to ethylene glycol in experimental animal studies are summarized below. Only statistically significant results are presented, unless specified otherwise. In some studies, statistical significance of certain findings was not presented by the authors. In such a case, the effects that were reported by the authors are presented in the table below without mentioning a P-value.

A total of 5 studies were on the effect of different cryoprotectants. The later were tested on Pacific white shrimp (*Litopenaeus vannamei*) [31], semen quality of the Oravka cock [32], blastocyte stage of mouse embryos [33], Holstein heifers and cows [34], and sheep ovarian tissue [35]. In total, the toxicity of 8 different cryoprotectants was tested: methanol [31], ethylene glycol (EG) [31, 32, 34, 35], ethylene glycol and sucrose (EG+SUC) [31], propylene glycol [31], glycerol [32], Glycerol-sucrose (GS) [33, 34], ethylene glycol-ficoll-sucrose (EFS40) [33], dimethyl sulfoxide (DMSO) [31, 32, 35], EG+DMSO [35]. Results showed that, for the Pacific white shrimp, ethylene glycol was the least toxic to shrimp embryos and larvae [31]. For the Oravka cock, higher ($P<0.05$) proportion of spermatozoa with damaged plasma membranes was found in the DMSO and EG groups and significant differences ($P<0.05$) in the numbers of live and necrotic spermatozoa

between GL and DMSO, EG groups were observed [32]. Blastocyte stage of mouse embryos showed a high recovery rate: EFS: 90% and GS: 85% [33]. The exposure of sheep ovarian tissue to EG, DMSO, and EG+DMSO significantly reduced the percentage of normal preantral follicles when compared with fresh ovarian tissue. Viability testing showed that, at Day 0, no morphologic differences among isolated follicles from all groups with EG or DMSO were seen as well as degeneration in group EG+DMSO cryopreserved at a greater frequency than those from the other groups. At Day 1, follicular viability was the same in fresh follicles and EG group and significant reduction of viability in group with DMSO and EG+DMSO. At Days 6 and 10, a decrease in the percentage of follicles considered viable was observed and a significant reduction of viability in group with DMSO and EG+DMSO [35]. In Holstein heifers and cows, it was concluded that no difference was observed in the pregnancy rate among the three cryoprotectants used in this study [34].

Other types of studies were performed with ethylene glycol. A non-guideline toxicity study on the effect of vitrification of human oocytes on pregnancies was also performed [36]. A high pregnancy rate was also observed with 100% of autologous oocytes with an average of 4.8 oocytes and with the patient with autoimmune disease (100% with an average of 5 oocytes). A low pregnancy rate was observed in the patients with menopause (53.8% with average of 3.9 oocytes) and premature ovarian failure (25% with average of 4.5 oocytes).

The effects of ethylene glycol on allotransplantation was also studied in a non-guideline toxicity study [37]. The latter tested the effects of allotransplantation of cryopreserved prepubertal mouse ovaries and its effects on restoring puberty, the fertility, and the methylation profile of Snrpn-DMR. Histology results showed no difference between the control and the two cryopreserved groups. The percentages of viable oocytes obtained from the ED20 group (87%) and the EG5.5/30 group(84.6%) were slightly lower than those of the fresh group (92.6%). The puberty assessment showed that the onsets of the oestrous cycle that occurred on days 10–14 post surgery of the two cryopreserved transplanted groups were longer than those of the fresh transplanted group. Morphology of the ovaries showed no significant differences in the number of various stages of follicles were observed between two cryopreserved and fresh transplanted groups. The analysis for fertility potential via IVF preparation showed no significant differences in morphology were observed in fresh and cryopreserved trans-planted groups. Additionally, it showed that the 2-cell embryos progressed to blastocysts at the same rate from two cryopreserved transplanted groups compared with controls and there was no significant difference in the two vitrified-warmed transplanted groups.

Another study looked at the effects of ethylene glycol on pregnancy rate of bovine embryo recipients [38]. Pregnancy rate was assessed in cows and heifers and reached from 33.3% to 59.2% depending on breed group.

One study not only looked at the different cryopreservatives but also included different buffers dromedary camel sperm: EY INRA-96® or

Green Buffer (GB) [39]. Cryopreservation was performed with buffer 6% (v/v) CPA (6% glycerol or ethylene glycol (EG) added 1:1 to give final concentrations of 3%) (FB) and catalase (500 IU/mL). Results of sperm kinematics show, in terms of CPA, before freezing and just after thawing: VCL (P = 0.001; P = 0.027), VAP and ALH were lower when CPA was not present. After 1.5 h, with EG present, ALH values were higher. Interactions between extender and CPA were observed at 0 h with STR, LIN and BCF and at 1.5 h with STR and LIN. At 0h, GB with CPA, and INRA with no CPA were the best combinations. After 1.5 h, INRA showed the lowest values in STR when no CPA present and the highest LIN when INRA contained EG.

9 References

1. ATSDR, *TOXICOLOGICAL PROFILE FOR ETHYLENE GLYCOL*. 2010, U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES; Public Health Service; Agency for Toxic Substances and Disease Registry.
2. NTP, *Monograph on the Potential Human Reproductive and Developmental Effects of Ethylene Glycol*. 2004, Center For The Evaluation Of Risks To Human Reproduction.
3. ECHA. *Substance information ethylene glycol*. 2022; Available from: https://echa.europa.eu/fr/substance-information/-/substanceinfo/100.003.159.
4. (NMAM), N.M.o.A.M., *GLYCOLS: METHOD 5523*. 1996.
5. Agency), E.U.S.E.P., *METHOD 8015C NONHALOGENATED ORGANICS BY GAS CHROMATOGRAPHY*. 2007.
6. Agency), E.U.S.E.P., *METHOD 8430 ANALYSIS OF BIS(2-CHLOROETHYL) ETHER AND HYDROLYSIS PRODUCTS BY DIRECT AQUEOUS INJECTION GAS CHROMATOGRAPHY/FOURIER TRANSFORMINFRARED*. 2014.
7. S, S., M. RK, and J. M., *Investigation of "crystallosis" in ethylene glycol toxicity.* , S.E.M. 8:555-562., Editor. 1975.
8. Kothandaraman, J. and D.J. Heldebrant, *Catalytic coproduction of methanol and glycol in one pot from epoxide, CO(2), and H(2).* RSC Adv, 2020. **10**(69): p. 42557-42563.
9. Engineering, C. *Ethylene Glycol Production*. 2015; Available from: https://www.chemengonline.com/ethylene-glycol-production/?printmode=1.
10. Australian government; Department of Climate Change, E., the Environment and Water. *Fact sheet Ethylene glycol (1,2-ethanediol)*. 2022; Available from: https://www.dcceew.gov.au/environment/protection/npi/substances/fact-sheets/ethylene-glycol-12-ethanediol.
11. Yue, H., et al., *Ethylene glycol: properties, synthesis, and applications.* Chemical Society Reviews, 2012. **41**(11): p. 4218-4244.
12. England, P.H. *Ethylene glycol Toxicological overview*. 2015; Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/455702/Ethylene_Glycol_PHE_TO_210815.pdf.
13. Slikker, W., Jr., et al., *Dose-dependent transitions in mechanisms of toxicity.* Toxicol Appl Pharmacol, 2004. **201**(3): p. 203-25.
14. Lamb Iv, J.C., et al., *Reproductive and developmental toxicity of ethylene glycol in the mouse.* Toxicology and Applied Pharmacology, 1985. **81**(1): p. 100-112.
15. Gulati, D.K., et al., *Ethylene Glycol: reproduction and fertility assessment in CD-1 mice when administrated in drinking water*. 1986, National Institute of Environmental Health Sciences.
16. DePass, L.R., et al., *Three-generation reproduction and dominant lethal mutagenesis studies of ethylene glycol in the rat.* Fundam Appl Toxicol, 1986. **7**(4): p. 566-72.

17. Hong, H.L., et al., *Comparative effects of ethylene glycol and ethylene glycol monomethyl ether exposure on hematopoiesis and histopathology in B6C3F1 mice.* J Environ Pathol Toxicol Oncol, 1988. **8**(7 Spec No): p. 27-38.
18. Harris, M.W., et al., *Assessment of a short-term reproductive and developmental toxicity screen.* Fundam Appl Toxicol, 1992. **19**(2): p. 186-96.
19. ECHA, *Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures*. 2017.
20. Bolon, B., et al., *Differential follicle counts as a screen for chemically induced ovarian toxicity in mice: Results from continuous breeding bioassays.* Fundamental and Applied Toxicology, 1997. **39**(1): p. 1-10.
21. Maronpot, R.R., et al., *Teratogenicity study of ethylene glycol in rats.* Drug Chem Toxicol, 1983. **6**(6): p. 579-94.
22. Price, C.J., et al., *The developmental toxicity of ethylene glycol in rats and mice.* Toxicol Appl Pharmacol, 1985. **81**(1): p. 113-27.
23. Price, C.J., et al., *DEVELOPMENTAL TOXICITY EVALUATION OF ETHYLENEGLYCOL (CAS No.107-21-1) IN CD RATS,* . 1988, NTP report.
24. Marr, M.C., et al., *Developmental stages of the CD (Sprague-Dawley) rat skeleton after maternal exposure to ethylene glycol.* Teratology, 1992. **46**(2): p. 169-81.
25. Neeper-Bradley, T.L., et al., *Determination of a no-observed-effect level for developmental toxicity of ethylene glycol administered by gavage to CD rats and CD-1 mice.* Fundam Appl Toxicol, 1995. **27**(1): p. 121-30.
26. Schuler, R.L., et al., *Results of testing fifteen glycol ethers in a short-term in vivo reproductive toxicity assay.* Environ Health Perspect, 1984. **57**: p. 141-6.
27. Tyl, R.W., et al., *Assessment of the developmental toxicity of ethylene glycol applied cutaneously to CD-1 mice.* Fundam Appl Toxicol, 1995c. **27**(2): p. 155-66.
28. Tyl, R.W., et al., *Developmental toxicity evaluation of ethylene glycol by gavage in New Zealand white rabbits.* Toxicological Sciences, 1993. **20**(4): p. 402-412.
29. Tyl, R.W., et al., *Evaluation of the developmental toxicity of ethylene glycol aerosol in the CD rat and CD-1 mouse by whole-body exposure.* Toxicological Sciences, 1995a. **24**(1): p. 57-75.
30. Tyl, R.W., et al., *Evaluation of the developmental toxicity of ethylene glycol aerosol in CD-1 mice by nose-only exposure.* Toxicological Sciences, 1995b. **27**(1): p. 49-62.
31. Dong, Q., J. Lin, and C. Huang, *Effects of cryoprotectant toxicity on the embryos and larvae of pacific white shrimp Litopenaeus vannamei.* Aquaculture, 2004. **242**(1-4): p. 655-670.
32. Svoradová, A., et al., *In vitro effect of various cryoprotectants on the semen quality of endangered Oravka chicken.* Zygote, 2017. **26**(1): p. 33-39.
33. Toodeh-Dehghan, F., et al., *Survivaland development of mouse 8-cells embryos after vitrification in glycerolsucrose and ethylen glycolbased solutions.* Journal of Veterinary Research, 2010. **65**(1): p. 25-30.

34. Dochi, O., K. Imai, and H. Takakura, *Birth of calves after direct transfer of thawed bovine embryos stored frozen in ethylene glycol.* Animal Reproduction Science, 1994. **38**(3): p. 179-185.
35. Tsuribe, P.M., C.A. Gobbo, and C. Landim-Alvarenga Fda, *Viability of primordial follicles derived from cryopreserved ovine ovarian cortex tissue.* Fertil Steril, 2009. **91**(5 Suppl): p. 1976-83.
36. Lucena, E., et al., *Successful ongoing pregnancies after vitrification of oocytes.* Fertil Steril, 2006. **85**(1): p. 108-11.
37. Wang, H.Y., et al., *Allotransplantation of cryopreserved prepubertal mouse ovaries restored puberty and fertility without affecting methylation profile of Snrpn-DMR.* Fertil Steril, 2013. **99**(1): p. 241-247.e4.
38. Nogueira, E., et al., *Effect of breed and corpus luteum on pregnancy rate of bovine embryo recipients.* Revista Brasileira de Zootecnia, 2012. **41**(9): p. 2129-2133.
39. Malo, C., E.G. Crichton, and J.A. Skidmore, *Preservation of the spermatozoa of the dromedary camel (Camelus dromedarius) by chilling and freezing: The effects of cooling time, extender composition and catalase supplementation.* Theriogenology, 2020. **153**: p. 9-18.

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