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Emmanuel Lemazurier(*), Anthony Lecomte, Franck Robidel and Frédéric Y. Bois.

Laboratoire de Toxicologie expérimentale, Institut National de l'Environnement Industriel et des Risques, INERIS DRC TOXI, Parc technologique ALATA BP2, F-60550 Verneuil en Halatte, France

(*) Corresponding author: E. Lemazurier, INERIS DRC/TOXI Parc technologique Alata BP 2 60550 Verneuil en Halatte, France tel +33 (0) 3 44 55 62 64, fax +33 (0)3 44 55 66 05, E-mail : emmanuel.lemazurier@ineris.fr

Propylene glycol monomethyl ether (PGME) is widely used as a solvent in numerous commercial products. Its chemical synthesis leads to the formation of 2 isomers, α and β, the later being usually present in the range of 0.5% to 1.5%. Isomer α has been shown to be of low toxicity. Isomer β raises concerns as to its reproductive and developmental effects.

We evaluated the reproductive and developmental toxicity of two different commercial mixes of PGME (Mix A 99% isomer α and 0.5% isomer β, Mix B 98.5% isomer α and 1.5% isomer β) on Sprague Dawley rats. The use of two mixes allowed us to differentiate between isomer α and isomer β effects. Male and female rats were exposed through drinking water to mixes A or B during a gametogenesis cycle (64 days for males and 15 days for females) to 0%, 2%, 5%, 10% and 15% (v/v) of each mix. These animals (F_0) and the three following generations (F_1, F_2 and F_3) were followed.

We observed a statistically significant decrease in the number of pups in isomer α treated-animals of generation F_1 and a non dose-related variation of the sex ratio in F_1 and F_2

generations after PGME mix B treatment. The most important effect obaerved was a decrease in testicular and epididymal sperm counts in relation to PGME isomer β in acute daily exposure, on the first and second parental generations. The effect evidenced on sex ratio needs further work in order to assay the potential persistent effects of PGME exposure.

Key words: *PGME isomers, reproductive toxicology, developmental toxicity.*

Running title: PGME reproductive toxicity E Lemazurier et al.

Introduction

Propylene glycol monomethyl ether (PGME) is a colorless liquid, miscible with water and many organic solvents. This makes it useful for a wide variety of solvent applications in the manufacture of lacquers, paints, dyes, inks, cleaning agents and liquid soaps, etc. Commercial PGME typically contains at least 98.5% 1-methoxy-2-propanol (α isomer), and only a small amount of 2-methoxy-1-propanol (β isomer) (Carney *et al*., 1999).

An ECETOC report has reviewed studies up to 1995 on different PGME mix effects on development and reproduction by the oral, inhalation and subcutaneous routes of exposure (European chemical Industry Ecology and Toxicology Center [ECETOC], 1995). Developmental studies with 100% of PGME isomer β evidenced in rats and rabbits different skeletal malformations and variations but not with PGME mix containing 99.5% isomer α. Reproductive effects were also assessed in a 2-generation-rats inhalation study conducted with 0, 300, 1000 and 3000 ppm of PGME (98% isomer α, 2% isomer β) for 6 hours/day, 5 days/week prior to mating and 6 hours/day, 7 days/week during mating, gestation and lactation (Carney *et al*., 1999). These concentrations corresponded to 0, 400, 1300 and 4000 mg/kg/day. That study did no evidence any treatment-related effects at 300 ppm of PGME. The no-observable-effect level for reproductive/neonatal effects was 1000 ppm, and that for parental toxicity was 300 ppm. The profile of generally low developmental or reproductive toxicity for PGME contrasts that of ethylene glycol monomethyl ether (EGME) and ethylene glycol monoethyl ether (EGEE), which are teratogenic and cause reproductive effects, and in particular testicular toxicity (Hardin, 1983; ECETOC, 1995). Two structural features seem to be key determinants of glycol ether reproductive and developmental toxicity (Carney *et al*., 2003): (1) carbon chain length (inversely related to toxicity), and (2) the presence of a primary alcohol moiety. Glycol ethers containing primary alcohol moieties (*e.g*. EGME) are metabolized by alcohol dehydrogenases to the corresponding alkoxy acids established as the

proximate developmental/reproductive toxicants (Hardin 1983; ECETOC, 1995). Glycol ethers containing secondary alcohol moieties (*e.g.*, PGME) are poor substrates for alcohol dehydrogenases and do not form appreciable quantities of alkoxy acid metabolites (Miller *et al*., 1983). Nevertheless, the PGME β isomer has been shown to be metabolized, into the corresponding alkoxy acid metabolite (Carney *et al*., 2003).

In order to further evaluate the potential reproductive and neonatal toxicity of two different commercial mixes of PGME (Mix A 99% isomer α and 0.5% isomer β, Mix B 98.5% isomer α and 1.5% isomer β) on Sprague Dawley rats, a 3-generation oral reproduction study was conducted. In addition to the standard parameters of a 2-generation test guidelines (U.S. EPA, 1999; OECD, 1983), behavior, demeanor, gross necroscopy, histology, weight, feed and water consumption, estrous cycle length and normality, litter size, sperm analysis, hormonal assay were also evaluated in adult and litters. Parental generation was exposed during a gametogenesis cycle and the effects on reproductive and developmental parameters of the F_0, F_1, F_2 and F_3 generations were evaluated.

Materials and methods

Test material

The PGME used in this study was obtained from Acros Organics (France). Two grades of purity were used: (1) PGME mix A: 99.5% propylene glycol monomethyl ether α and 0.5% propylene glycol monomethyl ether β and (2) PGME mix B: 98.5% propylene glycol monomethyl ether α and 1.5% propylene glycol monomethyl ether β.

Animals

The study was conducted in accordance with French State Council guidelines for the care and use of laboratory animals (decree n°87-849, October the 19th 1987) and was approved by the Institutional Animal Care and Use Committee at the INERIS. Animals were housed in the INERIS animal-care unit, a facility accredited by the Departmental Direction of Veterinary Services. Animals were kept in a HEPA-filtered, mass air-displacement room with a 12-h light-dark cycle at 20-24°C and relative humidity of 40-70%. Animals had access *ad libitum* to deionized water and rodent chow (AO4C10, UAR, Epinay sur Orge, France). Time-mated, 6- to 7-week-old, nulliparous Sprague Dawley male and female rats were obtained from IFFA CREDO laboratories (L'Arbresles, France). Animal allocation to treatment groups was done with body weight randomization to ensure unbiased weight distribution among groups. Animals were group-housed, up to 6 per cage, by treatment in polycarbonate cages Makrolon type 3 (UAR, Epinay sur Orge, France).

Experimental design

Groups of 13 male and 13 female rats were exposed to 0%, 2%, 5%, 10% and 15% PGME mix A or B (v/v) via drinking water for one gametogenesis cycle (64 days for males and 15 days for females respectively). Bottles were weighted each week in order to calculate the

quantity of each mix drank by the rats. Treatment of the first parental generation F_0 rats, called P_1 when bred, began at approximately 6 weeks of age. After exposure, P_1 rats were mated (one male:one female) to produce the F_1 litters. Ten males and females per group were randomly selected from the F_1 weanlings and assigned to new groups to become the second parental generation (P_2). This second parental generation and followings were not treated. The P_2 adults were then mated to produce the F_2 litters. Ten males and females per group were randomly selected from the F_2 weanlings and assigned to new groups to become the third parental generation (P_3). The P_3 adults were then mated to produce the F_3 litters which were sacrificed on gestational day 19.

Breeding procedures

Breeding of the F_0/P_1 adults started after 64 days (for males) and 15 days (for females) of treatment. Each female was placed with a single male from the same exposure group (10 females and 10 males) or with a non-exposed partner (3 treated females and 3 non-treated males or 3 non-treated females and 3 treated males) until pregnancy occurred or 2 weeks had elapsed. During each breeding period, daily vaginal lavage samples were evaluated for the presence of sperm, as an indication of mating. The day on which sperm were detected or a vaginal plug was observed *in situ* was considered as day 0 of gestation. If mating did not occur during the 2 weeks, the animals were separated without further opportunity to mate. For the F_1/P_2 and F_2/P_3 mating, cohabitation of male and female littermates was avoided. Three males and females from F_1/P_2 and F_2/P_3 litters born from treated groups were mated with non-exposed females and males, respectively.

Parental data

All adult rats were observed daily for changes in behavior, demeanor, or overt indications of toxicity. Rats found dead or moribund were submitted to a complete pathologic examination in an effort to determine the cause of death. F_0/P_1 rats were weighted weekly during the treatment period. Feed and water consumption were measured, the latter allowing the calculation of an ingested dose. Estrous cycle length and normality were evaluated daily by vaginal lavage (Cooper *et al*., 1993) for all F_0/P_1, F_1/P_2 and F_2/P_3 females starting at the beginning of the cohabitation period of the F_1/P_2, F_2/P_3, and F_3.

Litter data

All litters were examined as soon as possible after delivery. The following parameters were recorded for each litter: total litter size on the day parturition was initiated (day 0), the number of live and dead pups on days 0, 1, 4, 7, and 21 postpartum, the sex and the weight of each pup on days 1, 4, 7, 14, 21 of lactation. Any visible physical abnormalities or changes in demeanor in the neonates were recorded during the lactation period. All pups found dead, or pups that were euthanized in moribund condition, were examined to the extent possible for defects and/or cause of death and preserved in neutral, phosphate-buffered 10% formalin.

Sperm analysis

Sperm analysis was performed on F_0/P_1, F_1/P_2 and F_2/P_3 males. Sperm motility was first assayed. A dilution medium was prepared as follows: . bovine serum albumin (BSA) was added at 0.5% w/v to medium 199 with Hank's salts and L-glutamine (Sigma, St Quentin Fallavier, France). After BSA was dissolved, the solution was brought to pH 7.4 with 0.1N NaOH. The left cauda epididymis from all males was used for sperm motility analysis. It was placed into a Petri dish containing 2 ml of warmed dilution medium (37°C) for 2 min on a

heating plate. The cauda was held with forceps and one stab was made with a scalpel blade. The Petri dish containing the concentrated sperm sample was gently swirled to evently distribute the non-diffused sperm. Ten µl of this solution was deposed on slide 80µ, double chamber (Hamilton-Thorne Research, Berverly, MA, USA) heating at 37°C on a heating plate. Sperm samples were then analyzed for motion using the HTM-IVOS (Hamilton-Thorne Research, Berverly, MA, USA, user's manual version 12) (Appendix 1). Motion parameters measured are given in Appendix 2.

The sperm count per gram of testis and epididymis was also evaluated. Left testis was used to determine that number. Testis tunica albuginea was removed and testicular parenchyma was weighted and re-suspended with 15 ml of SMT medium (0.9% NaCl, 0.01% methiolate, 0.05% triton X-100). The mix was then crushed with an Ultra Thurax, medium speed during 30 seconds, twice. The solution was waved with a Vortex at maximal speed during 1 minute before a 1 ml sample of the solution was placed for 1 minute in an ultra sound bath in order to desegregate possible remains. After a new agitation with the vortex at medium speed during 30 seconds twice, 15 µl were deposited on a Malassay cell for reading under microscope with a 10 x lens. The epididymis was treated in the same way as the testis.

Hormonal assays

Testosterone (T) and 17β-estradiol (E2) levels were assayed with Enzyme Linked ImmunoSorbent Assay technique (ELISA) according to supplier protocol (R et D System, Lille, France). Intra-assay and inter-assay precision were 6.7% and 8%, respectively for E2 and 9.5% and 11.7%, respectively for T. The linearity calculated by (observed hormonal level/expected hormonal level) x 100 after dilutions of one assayed neat sample was 98% for E2 and 96% for T. The sensitivity of the assays was 10.1 pg/ml for E2 and 3.8 pg/ml for T.

Statistical evaluation

Statistical significance was performed using the S-PLUS2000 software. Parental data were analyzed by ANOVA linear regression. Lost, pre-coital delay and pups number values were regressed on isomers α or β PGME concentrations. Sex ratio differences were tested using a binomial distribution test (Steel and Torrie, 1960)

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Results

PGME intake

Table 1 gives the calculated isomer α PGME and isomer β PGME dose ingested by F_0/P_1 generation treated with PGME mix A and mix B. The total dose of isomer α PGME ingested by rats treated with PGME mix A and mix B was about the same (Table 1A and 1B). As expected, the dose of isomer β PGME was higher for rats treated with PGME mix B (Table 1A and 1B) than those treated with PGME mix A.

Parental data

The main effects observed on F_0/P_1 males and females were a decrease in weight (20% to 50%) related to a decrease in food intake (data not shown). Meanwhile, F_0/P_1 females treated with a mix of 3,350 mg/kg/d of PGME alpha and 16.75 mg/kg/d of PGME beta or 3,940 mg/kg/d of PGME alpha and 19.70 mg/kg/d of PGME beta (mix A) and with a mix of 3,430 mg/kg/d of PGME alpha and 51.45 mg/kg/d of PGME beta or 3,700 mg/kg/d of PGME alpha and 55.50 mg/kg/d of PGME beta (mix B), had also smaller litters (Figures 1 and 2), statistically significant only in relation to isomer α PGME. Only one pup was present in F_0/P_1 females treated with a mix of 3,940 mg/kg/d of PGME alpha and 19.70 mg/kg/d of PGME beta (mix A). We also noticed an increase in pre-coital delay in F_0/P_1 animals treated with a mix of 3,430 mg/kg/d of PGME alpha and 51.45 mg/kg/d of PGME beta (mix B) and a mix of 3,940 mg/kg/d of PGME alpha and 19.70 mg/kg/d of PGME beta (mix A) and 3,700 mg/kg/d of PGME alpha and 55.50 mg/kg/d of PGME beta (mix B), statistically significant only in relation to isomer α PGME (Figures 1 and 2). No change in number of lost pups was noticed in F_0/P_1 generation (Figures 1 and 2). The effects observed appear at lower PGME beta concentrations than PGME alpha concentrations. Parents of the F_1/P_2 and F_2/P_3 generations did not show any trouble (both F_1/P_2 male and female issued from F_0/P_1 treated animals or

only F_1/P_2 male or female issued from F_0/P_1 treated animals bred with a non treated animal). Those generations did not have a 15% PGME mix A treated group because of the mortality observed in this group in F_0/P_1 generation.

Litter data

A diffuse effect on the distribution of male and female pups and on sex ratio was observed. Even though no statistical effect was evidenced using a binomial distribution test, the sex ratio was inverted in both F_1/P_2 and F_2/P_3 generations without any relation to treatment when both F_0/P_1 male and female as well male or female were treated with mix A or B PGME. Due to a technical problem, we did not register sex ratio data concerning the animals issued from F_0/P_1 females treated with PGME 5% and bred with a non-treated male. But that effect was not significant for none of the treated groups (data not shown).

The main observed effect was a decrease in the number of male pups per litter, directly responsible in most cases of the inversion of the sex ratio. We also observed a decrease in both male and female numbers per litter as well as an increase of female number, without inversion of the sex ratio. None of these effects evidenced any dose relation. But that effect was not significant for none of the treated groups (data not shown).

Sperm analysis

The sperm count per gram of epididymis in F_0/P_1 generation was strongly disturbed by beta PGME treatment (Figures 3 and 4).

In F_0/P_1 epididymis, we evidenced a statistically significant dose-related decrease in sperm count of 56% in animals treated with 1,120 mg/kg/d of PGME alpha and 16.80 mg/kg/d of PGME beta (mix B 2%), 79% in animals treated with 2,190 mg/kg/d of PGME alpha and 32.85 mg/kg/d of PGME beta (mix B 5%), 80% in animals treated with 3,620 mg/kg/d of

PGME alpha and 54.30 mg/kg/d of PGME beta (mix B 10%), 76% in animals treated treated with 4,360 mg/kg/d of PGME alpha and 21.80 mg/kg/d of PGME beta (mix A 15%) and 70% in animals with 5,120 mg/kg/d of PGME alpha and 76.80 mg/kg/d of PGME beta (mix B 15%), compared to control. No significant effect was observed in F_0/P_1 testis (Figures 3 and 4). No significant effect was observed in F_1/P_2 or F_2/P_3 epididymis or testis (Data not shown). Data collected on percentage of motile sperm, VCL, VAP, VSL did not show any variations in any of treated group when compared to control.

Hormonal assay

Hormonal assays did not show any variations in estradiol or testosterone concentration in F_0/P_1, F_1/P_2 male or female groups (data not shown).

Discussion

The goal of this work was to evaluate effects of 2 mixes of commercial PGME (99.5% isomer α, 0.5% isomer β and 98.5% isomer α, 1.5% isomer β) on rat reproductive and developmental features. We chose a 3-generation test study in which only the first parental generation (F_0/P_1) was treated during a whole gametogenesis cycle in order to evaluate possible persistent effects. We chose to treat animals *via* drinking water to ensure that the treatment continuously covered a whole gametogenesis cycle, 24 h a day, 7 days a week. In the case of rats, a cycle of gametogenesis extends on approximately 60 days in male and 15 days in female (OCDE, 1998). Isomer α and β PGME intake by rats were obtained from measurements of water consumption. Even though the chosen PGME concentrations were high (between 1,000 and 5,000 mg/kg/day for PGME α and 5 to 8 mg/kg/day for PGME β), they were still in accordance with those used in previous works (ECETOC report 1995). These exposure doses are much higher than those encountered during occupational exposures (about 2 mg/kg/day of PGME α according to Devanthery et al., 2000). PGME β typical exposure should be about a hundred times lower since PGME mixes contains only 0.5 to 1.5% isomer β.

Among the tested generations, F_0/P_1 was the most affected, showing a decrease in litter size above about 3,000 mg/kg/d PGME α and 16.75 mg/kg/d PGME β (Figures 1 and 2). This generation also evidenced an increase in pre-coital delay above about 3,000 mg/kg/d PGME α and 19 mg/kg/d PGME β. The decrease in litter size was not related to a post-partum effect since number of lost pups did differ between the various PGME dose groups assayed after birth. This decrease in litter size was therefore related to implantation loss. These results are in accordance with numerous publications on the subject (ECETOC, 1995).

In Carney *et al*. (1999) sex ratios did not differ statistically between controls and litters issued from parents treated with 0, 400, 1300 and 4000 mg/kg/day of PGME (98% isomer α,

2% isomer β) prior to mating and during mating, gestation and lactation. In our work, the sex ratio of F_1/P_2 and F_2/P_3 litters was also not affected. However, when both parents or one of the parents were treated with PGME mix A or B a slight but non-significant increase in the number of males in the litters was observed. If this effect were to be confirmed, it would imply that treatment with PGME α and β mixes have a specific effect on the number of male in litters, directly related to the sex chromosome. So far, no evidence has been provided of a specific effect of PGME or any glycol ethers on sex chromosomes. El Zein *et al*. (2002) show a genotoxic effect of EGME in human: Mexican children, issued from women strongly exposed to EGME before or during pregnancy, evidenced the same malformations than those observed in Turner syndrome. The X chromosome has been shown to be responsible for this syndrome (Noonon, 1994). Furthermore, the observed malformations were strongly correlated to chromosomal aberrations in blood cells. It would be of interest to check whether PGME α and β mixes could be responsible for X chromosome aberrations leading to the increase of male numbers in subsequent generations.

The most relevant results of our work came from sperm analysis. We showed a large decrease (> 50%) in sperm counts in epididymis of F_0/P_1 generation treated with PGME mix α and β (Figures 3 and 4). Sperm count in F_0/P_1 epididymis decrease was statistically significant for PGME isomer β doses as low as 14.55 mg/kg/d and this effect was related to treatment. We did not use data on percentage of progressively motile sperm, ALH, BCF, linearity and straightness because these have been shown to be no relevant in toxicology study (Kato *et al*., 2001). The mechanism could involve the bioconversion of isomer β into MPA (Carney *et al*., 2003) and raises concern about MPA persistance and reprotoxicity. Carney *et al*. (2003) showed that, in rabbits, PGME isomer β was totally and rapidly bioconverted into 2-methoxypropionic acid (MPA), which in turn had a relatively long elimination half time (33-44 hours). Our results show that high daily exposure to a commercial PGME formula is

harmful to male sperm in rats, and that this effect is directly related to the small amount (0.5% or 1.5%) of isomer β in the mixture.

In this work, we found a NOEL of 11.5 mg/kg/d for PGME β-induced sperm count decrease in rats in case of a continuous exposure to "commercial pure" PGME (isomer α 99.5%; isomer β 0.5%). The suspected effect on the sex ratio will require further work in order to assess the possibility of persistent effects of PGME exposure through a genotoxic mechanism.

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