

# Reproductive Toxicity of Ethylene Glycol Monoethyl Ether Tested by Continuous Breeding of CD-1 Mice

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The reproductive toxicity of ethylene glycol monoethyl ether (EGEE) was evaluated in the Fertility Assessment by Continuous Breeding protocol. Both male and female CD-1 mice were given 0, 0.5, 1.0 or 2% EGEE in the drinking water and were housed as breeding pairs continuously for 14 weeks. Significant adverse effects on fertility were seen at 1 and 2% but not at 0.5%. After the continuous breeding phase of this test was completed, treated males were housed with control females and treated females with control males and fertility and reproduction were compared to the corresponding pairs of control male and control female mice. Both males and females from the 1 and 2% groups were affected. Testicular atrophy, decreased sperm motility and increased abnormal sperm were noted in the treated males, but no specific anomalies were detected in the females.

## Introduction

Ethylene glycol monoethyl ether (EGEE, 2-ethoxyethanol) is a glycol ether with widely applicable solvent properties. It is used in a variety of industrial applications and in products used by consumers, including paints and cleaners. NIOSH has estimated that, between 1972 and 1974, about 400,000 workers were exposed to EGEE (1). Previous studies have demonstrated that EGEE exposure caused testicular atrophy in males and birth defects in offspring exposed to EGEE *in utero* (2,3). No studies have been found in the published literature which describe fertility in animals after EGEE exposure.

The National Toxicology Program has developed and is validating a new reproductive toxicity testing system. This test system is designated Fertility Assessment by Continuous Breeding (FACB). It is composed of up to five interrelated tasks including: task 1, dose finding; task 2, continuous breeding; task 3, identification of the affected sex; task 4, offspring assessment; and task 5, hormone measurements. The new system provides an alternative to multigeneration studies and produces similar comprehensive reproductive toxicity data at a lower cost and in a shorter time frame.

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EGEE was studied for reproductive toxicity by using the FACB protocol. Male and female CD-1 mice were treated in task 2 with either 0, 0.5, 1.0 or 2% EGEE in the drinking water throughout the course of the study. The mice were monitored for effects on fertility, litter size, offspring survival and pup weight. After the continuous breeding portion of the study, the high dose treated males were cohoused with control females and high dose treated females with control males. The mating performance in that crossover mating portion of the study was used to determine whether males, females or both sexes were affected by the chemical exposure under the conditions of the study.

## Materials and Methods

### General Study Design

The flow chart for the NTP Fertility Assessment by Continuous Breeding Protocol is shown in Figure 1. Mice are quarantined for 2 weeks prior to task 1 or for 5 weeks prior to task 2. Task 1 utilizes a vehicle control and five dose groups (8 males and 8 females per group) and employs a 14-day repeated dose or continuous administration schedule in order to ascertain the dose levels to be administered in task 2. Task 1 is not performed if adequate data are available in the literature to select task 2 dose levels.

Task 2 is the continuous breeding phase of the

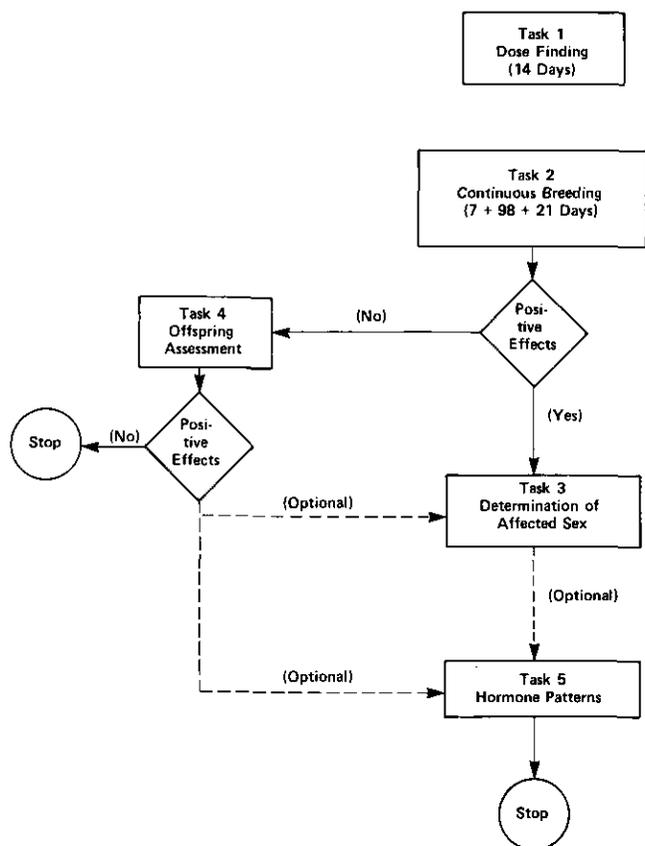


FIGURE 1. Flow chart for NTP fertility assessment by continuous breeding protocol.

protocol. This phase employs a vehicle control group (40 males and 40 females) and three dose groups (20 males and 20 females per group). The high dose may produce some significant signs of toxicity, but should not suppress body weight gain more than 10% compared to controls and should allow 90% or greater survival in male and female CD-1 mice. The mid-dose should produce minimal or no toxic effects and the low dose ideally is a no effect dose level. Eleven-week-old male and female CD-1 mice are exposed to the chemical during a 7-day pre-mating period, after which they are randomly paired (one male:one female) within each dose group and cohabited for 98 days (14 weeks). New-born litters are evaluated and immediately sacrificed. Chemical exposure is continuous during the 98-day cohabitation period and the 21-day segregation period which follows. The final litters born to the control and high dose pairs during the 21-day segregation period may be weaned and evaluated for reproductive performance in task 4 (Offspring Assessment). Chemical exposure is continued for the offspring.

If significant effects on reproductive performance (fertility or litter size) are found in task 2, then the affected sex (male, female or both) is determined by performing task 3. Task 2 high dose males are randomly paired with control females, high dose females with

control males, and control males with control females (20 pairs per group; all controls are assigned new cagemates). The pairs are cohabited for 7 days, fewer if a copulatory plug is observed. Considering the crossover breeding design, the chemical treatment is not feasible during the 7-day cohabitation. Reproductive performance is determined by evaluation of the litters and by effects on reproductive organs.

If minimal or no reproductive effects are noted in task 2, then reproductive performance in the control and high dose offspring from the final task 2 litters is evaluated (task 4). Chemical exposure continues throughout task 4. At  $70 \pm 10$  days of age, 20 high dose male offspring are randomly paired with 20 high dose female offspring and 20 control male offspring are randomly paired with 20 control female offspring (one pair per cage). If possible, all high dose and control task 2 litters are sampled and sibling matings are avoided. The pairs are cohabited for 7 days or fewer if a copulatory plug is observed. Chemical administration is continued during cohabitation. Reproductive performance is determined by evaluation of the litters. If reproductive performance is adversely affected in the high dose pairs, then task 3 can be carried out with these same mice to determine whether one or both sexes are affected. In this instance, the high dose animals are bred to the opposite sex control animals.

## Chemical

Ethylene glycol monoethyl ether (EGEE, CAS No. 110-80-5) was obtained from Union Carbide. Chemical purity and dosing solution stability were analyzed by the Midwest Research Institute under contract to the National Toxicology Program. The chemical was 99.4% pure with one major impurity which was 0.59% and three minor impurities totaling 0.03%.

## Dosage Formulation and Analysis

EGEE dosing solutions were prepared at 0.5, 1.0 and 2.0% (w/v). Dosing solutions were prepared fresh once every 2 weeks and stored at room temperature under yellow safe lights. The referee analyses were performed every 6 weeks. Referee analyses indicated that dosage solutions were within 99 to 108% of the intended dosage concentrations.

## Animals

COBS CrI:CD-1, (ICR)BR outbred albino mice (6 weeks of age) were purchased from Charles River Breeding Laboratories, Inc., Kingston, NY. During the 5-week quarantine period, two males and two females were sacrificed and their sera evaluated for antibodies against 11 mouse viruses (Microbiological Associates, Inc., Bethesda, MD). All sera were negative for viral antibodies. Furthermore, fecal samples were collected from two randomly selected mice and examined for

endoparasites. All results were negative. All study animals were individually identified with metal ear tags and assigned to control or treated groups using a stratified randomization procedure based on body weights.

Males and females were originally group housed by sex in solid-bottom polycarbonate cages with stainless steel wire lids, two per cage during quarantine and the 1-week pre-mating period. Subsequently, the animals were housed as breeding pairs or individually. Ab-Sorb-Dri bedding (Laboratory Products, Inc., Garfield, NJ) was used in all cages. Distilled water and Purina certified ground rodent chow (#5002) were provided *ad libitum*. Automatically controlled photoperiods were 14 hr light/10 hr dark (lights on 0700 to 2100 hours). Temperature was maintained at  $23 \pm 2^\circ\text{C}$  and relative humidity at  $45 \pm 25\%$ . Cages were sanitized weekly by using A33 detergent (Airwick Professional Products Division, Secaucus, NJ) and  $180^\circ\text{F}$  water.

### Clinical Signs, Body Weights and Feed Consumption (Tasks 2 and 3)

Cage-side observations for clinical signs and litters were made twice daily and the animals were given a complete physical examination at the weekly weighing. Body weights were determined weekly and at necropsy. Water consumption was measured gravimetrically at weekly intervals.

### Fertility and Reproductive Performance (Tasks 2 and 3)

During the 14-week cohabitation period and the subsequent 3-week segregation period, the number and proportion of fertile pairs and the number of litters produced per breeding pair were determined. In addition, for all litters in tasks 2 and for the one litter per pair in task 3, the number and proportion of live pups per litter, sex of live pups (males/total) per litter, and mean body weight of live pups per litter were determined. In particular, the live offspring were sexed, counted, pooled and weighed by sex; dead pups were counted, but not weighed or sexed. The pups were immediately sacrificed by decapitation, thereby allowing the pairs to breed again at postpartum estrus. Breeding pairs were defined as fertile if they produced at least one pup (dead or alive).

Following delivery of the final litter during the 3-week segregation period, the females in the 0.5% EGEE group were sacrificed and discarded. The males in this group were sacrificed and discarded at the conclusion of the 14-week cohabitation period.

Since adverse effects on fertility and reproduction were noted in the 1 and 2% EGEE groups (task 2), task 3 was performed in order to determine whether one or both sexes were affected. On the day following the 3-week segregation period (task 2), the males exposed

to 2% EGEE were randomly paired with control females, and control males were randomly paired with females exposed to 2% EGEE. In addition, a third group was formed by randomly pairing control males with control females. Treatment with EGEE was discontinued during the 1- to 7-day cohabitation period. The females were checked twice daily for copulatory plugs. Upon detection of a plug or after 7 days (whichever occurred first), the male and female mice were separated, housed individually, and treatment resumed, (i.e., control or treated). The number and proportion of positive matings were recorded. During the 3-week period following the 1-week mating trial, the number and proportion of fertile pairs, mean litter size, sex, number and proportion of live pups and mean pup weight per litter were determined. Exfoliative vaginal cells were sampled for five consecutive days following task 3 from the females which were not impregnated and/or did not deliver any pups animals to determine if the females were exhibiting normal estrous cycles. The vaginal smears were stained with Toluidine Blue O and cell types determined by light microscopy.

Five weeks after the one-week mating trial all male and female breeding partners were weighed and then asphyxiated with  $\text{CO}_2$  and bled by cardiac puncture. The left testis removed and weighed with the epididymis intact. Paired ventral prostate and paired seminal vesicle/coagulating glands were removed and weighed and fixed in 10% neutral buffered formalin. The right testis was separated from the epididymis and both organs were weighed separately. The gonads were fixed in Bouin's fixative. The right caudal epididymis was excised and weighed and sperm motility, cauda epididymal sperm counts and percent abnormal sperm were measured. For females, the intact reproductive tract (ovaries, oviducts, uterus and upper half of vagina) were excised and weighed. The reproductive tract was preserved in neutral buffered 10% formalin. After removing the top of the cranium, the entire head (males and females) was placed in 10% formalin in order to fix the brain and pituitary *in situ*. Subsequently, the brain (cerebrum, cerebellum and medulla) and pituitary were dissected from the cranium and weighed. The liver was weighed and discarded.

### Statistical Analysis

Statistical analyses of the data were performed by Program Resources, Inc. (Research Triangle Park, NC) using software written in APL and executed in an NIH DEC 10 computer.

The chi-square test for homogeneity was used to test for the joint equality of the proportion of fertile pairs, the proportion of females with copulatory plugs (positive matings), and the proportion of fecund females among treatment groups (4). Pairwise comparisons were performed by using Fisher's exact test.

The number of litters and the number of live pups per litter were computed on a per fertile pair basis and then

treatment group means determined. The groups were compared overall using the chi-square approximation to the Kruskal-Wallis test. Pairwise comparisons of treatment groups then were performed using the normal approximation to the Mann-Whitney *U* test (4). The analysis was carried out both on male and female data combined, and separated by sex.

The number of pups born alive over the total number of pups produced by each pair was defined as the proportion of live pups. The sex of live pups was expressed as the proportion of male pups born alive out of the total number of live pups born to each fertile pair. The sex of the pups born dead was not determined. The chi-square approximation to the Kruskal-Wallis test was performed to determine whether differences existed among treatment groups for the proportion of live pups and the sex of live pups (males/total) per litter. The normal approximation to the Mann-Whitney *U* test was used to make pairwise comparisons (4).

The Kruskal-Wallis and Mann-Whitney *U* tests also were employed to test for among group and pairwise differences in average live pup weight per litter. In addition, an analysis of covariance was performed for average live pup weight per litter with treatment group as the classification variable and average number of live and dead pups per litter (task 2) or number of live and dead pups per litter (task 3) as the covariate. Least-squares treatment group means were generated from the analysis of covariance and these were tested for pairwise equality using a one-tailed *t* test (5). To control for sex of live offspring both the nonparametric and parametric analyses were performed separately for both male and female data combined.

An analysis of variance was used to determine whether the treated and combined control group body weights differed significantly. The chi-square approximation to the Kruskal-Wallis test was employed to

determine whether differences existed between the treated and combined control group organ weights.

The overall comparisons made with the chi-square and *F* distributions were two-sided, while the pairwise comparisons made with the normal and *t* distributions were one-sided.

## Results

No litters were found during the continuous breeding phase of this study when the males and females received 2% EGEE in the drinking water (task 2, Table 1). The effects on reproduction were observed at dosage levels which did not produce detectable signs of clinical toxicity or adverse body weight effects in the mice. Two of the 20 pairs which received 1% EGEE did not deliver any litters during this phase of the study. All pairs in the control and 0.5% EGEE groups had at least one litter in task 2.

There was a decrease in the mean number of litters at the 1% EGEE level (Table 2). The number of live pups per litter was reduced and the proportion of pups born alive, and mean live pup weight were also significantly reduced in the 1% group compared to the controls. The animals in the 0.5% treatment group did not seem to be adversely affected by the EGEE exposure with respect to these endpoints.

The continuous breeding portion of the protocol, task 2, generates a considerable amount of data on the reproductive performance of the treated pairs, but it cannot discriminate which sex (or sexes) may be affected by the chemical exposure. Therefore task 2 is interfaced with a crossover mating study, task 3, designed to identify the sex affected by the chemical exposure. In cases where significant effects on fertility are not apparent (e.g., ethylene glycol, unpublished observations), the offspring from the treated pairs would be studied for reproductive performance in task 4.

In this particular case both the 2% and the 1% animals were tested in a crossover mating trial to determine whether the males or females or both sexes had compromised reproductive performance when matched with control animals. At 2% EGEE in the drinking water, the treated females had no fertile matings when they were cohabited with control, known-fertile male CD-1 mice, despite the fact that at least seven of the pairs had copulated during the 1-week cohabitation (Table 3). The males which were treated

**Table 1. Ethylene glycol monoethyl ether: fertility of pairs during continuous breeding.**

Group	No. fertile/no. cohabited	Fertility index, % <sup>a</sup>
Control	40/40	100
0.5%	20/20	100
1.0%	18/20	90
2.0%	0/20*	0*

<sup>a</sup>Fertility index (%) = (No. fertile/no. cohabited) × 100.

\**p* < 0.001 as compared to controls.

**Table 2. Ethylene glycol monoethyl ether: reproductive performance during continuous breeding.**

Variables	EGEE dose level			
	0.0	0.5%	1.0%	2.0%
Litters per fertile pair <sup>a</sup>	4.5 ± 0.1	4.8 ± 0.1	3.0 ± 0.3*	—
Live pups per litter <sup>a</sup>	9.8 ± 0.5	9.3 ± 0.6	2.6 ± 0.4*	—
Proportion of pups born alive <sup>a</sup>	0.94 ± 0.03	0.95 ± 0.02	0.50 ± 0.7*	—
Live pup weight, g <sup>a</sup>	1.63 ± 0.02	1.64 ± 0.02	1.59 ± 0.04*	—

<sup>a</sup>Values are group mean ± standard error.

\**p* < 0.05 as compared to controls.

with 2% EGEE and were cohabited with control females had significantly fewer fertile matings than the control pairs (28% as compared to 85%, respectively) (Table 3) and number of live pups per litter was slightly decreased from 6.7 in the control group to 5.4 for the litters sired by the treated males. There was no effect on the number of dead pups per litter (Table 4).

The fertility of the mice from the middle dose group (1% EGEE) was also tested by matching the treated males and females with controls for a 1-week cohabitation period. The standard protocol does not call for this extra testing of the second dose level, and generally there are not enough animals to accommodate this additional test. In this case, however, control mice were reused after the 2% EGEE crossover mating had ended, and the controls, the 1% group and 2% group mice were all necropsied after the second crossover mating study. This approach allowed us to study fertility and reproduction in the intermediate dose level. There was a decrease, though not statistically significant, in the percent fertile matings for both the treated males cohabited with control females (44%) and the treated females cohabited with control males (53%) compared to the control pairs (78%, Table 5). The number of live pups per litter was slightly lower in the treated female  $\times$  control male group than in the control female  $\times$  control male group; pup weight also seemed to be decreased in that treated group. Significant effects were not demonstrated in the mid-dose treated male  $\times$  control female mating (Table 6).

Since there were significant effects on fertility and reproduction in both treated males and females, all animals were necropsied and reproductive tract and gonadal tissues were weighed and examined for gross and histological effects. EGEE was related to profound dose-related decreases in sperm motility and increases

**Table 3. Ethylene glycol monoethyl ether: fertility in crossover mating study (high dose, 2%).**

	Control $\delta$ $\times$ control $\text{♀}$	Control $\delta$ $\times$ high dose $\text{♀}$	High dose $\delta$ $\times$ control $\text{♀}$
No. fertile	17	0	5
No. cohabited	20	15	18
Fertility index (%)	85	0*	28*

\* $p < 0.05$

**Table 4. Ethylene glycol monoethyl ether: reproductive performance during crossover mating study (high dose, 2%).**

	Control $\delta$ $\times$ control $\text{♀}$	Control $\delta$ $\times$ high dose $\text{♀}$	High dose $\delta$ $\times$ control $\text{♀}$
Live pups per litter <sup>a</sup>	6.7 $\pm$ 0.8	— <sup>b</sup>	5.4 $\pm$ 1.8*
Dead pups per litter <sup>a</sup>	0.1 $\pm$ 0.1	—	0
Live pup weight, g <sup>a</sup>	1.73	—	1.76

<sup>a</sup>Values are mean  $\pm$  standard error.

<sup>b</sup>Seven vaginal plugs detected, but no fertile matings.

\* $p < 0.05$ .

**Table 5. Ethylene glycol monoethyl ether: fertility in crossover mating study (middle dose, 1%).**

	Control $\delta$ $\times$ control $\text{♀}$	Control $\delta$ $\times$ mid dose $\text{♀}$	Mid dose $\delta$ $\times$ control $\text{♀}$
No. fertile	14	10	8
No. cohabited	18	19	18
Fertility index, %	78	53	44

in the percent of morphologically abnormal sperm (Table 7). Cauda epididymis weight and cauda epididymal sperm counts were reduced but the pairwise comparisons were not statistically significant. Treatment-related lesions were identified in the testis, including decreased testis weight and decreased spermatogenesis. The dose-related decrease in spermatogenesis conformed with findings of testicular atrophy. No gross or microscopic lesions were significantly increased in the female mice.

## Discussion

The findings from the continuous breeding phase of these investigations indicated that EGEE caused a profound effect on reproductive function in CD-1 mice both at the 1 and 2% dose levels in the drinking water. The design of the task 2 protocol is such that the complete absence of fertile matings in the breeding pairs treated with 2% EGEE could not be attributed to effects on either sex to the exclusion of the other, since both male and female mice received the same concentration of EGEE in the drinking water. Therefore, the crossover mating trial, task 3, was performed to determine whether both sexes were affected by EGEE ingestion or if one sex was significantly more susceptible to the chemical toxicity. The results of the crossover mating trial indicated that EGEE did not affect reproduction in one sex, to the exclusion of affecting the other. At 2% EGEE, the females had no fertile matings while the males had 28% fertile matings. Although it seems that the female was more susceptible to the effects of EGEE than the male under the same exposure conditions, the relative toxicity is certainly a minimal distinction where both were significantly affected by the chemical at similar dosages

Table 6. Ethylene glycol monoethyl ether: reproductive performance during crossover mating study (middle dose, 1%).

	Control ♂ × control ♀	Control ♂ × mid dose ♀	Mid dose ♂ × control ♀
Live pups per litter	5.7 ± 1.2	4.2 ± 0.9*	5.1 ± 1.2
Dead pups per litter	0.3 ± 0.2	0.8 ± 0.4*	0.4 ± 0.3
Live pup weight, g	1.69	1.59	1.62

\**p* < 0.05.

Table 7. Ethylene glycol monoethyl ether: sperm assessment in treated mice.

	EGEE dose levels		
	Control	1%	2%
Sperm/mg epididymis <sup>a,b</sup>	729 ± 51	633 ± 66	593 ± 61
Percent motile <sup>a</sup>	68 ± 4	64 ± 4	42 ± 8*
Percent abnormal <sup>a</sup>	3.3 ± 1.0	8.2 ± 1.6*	43 ± 8*
Testis weight, mg <sup>a</sup>	136 ± 4	121 ± 5	88 ± 7*
Epididymal weight, mg <sup>a</sup>	62 ± 1	62 ± 2	51 ± 2
Caudal weight, mg <sup>a</sup>	22.4 ± 1	20.1 ± 1	19.4 ± 1

<sup>a</sup>Values are mean and standard error.<sup>b</sup>Million sperm per gram cauda epididymal tissue.\**p* < 0.05 compared to controls.

and the no observed effect level for both sexes seemed to be about 0.5% in the drinking water.

The results which have been described from this new testing system are consistent with previous investigations on the male reproductive toxicity of EGEE. For example, testicular atrophy has been observed in male mice exposed to EGEE (2). In that study, male mice received ethylene glycol, EGEE or five other monoalkyl glycol ethers at levels from 62.5 to 4000 mg/kg/day by gavage 5 days per week for 5 weeks. That study showed that EGEE was associated with a significant decrease in testis weight at a daily dose level of 1000 mg/kg but accessory sex gland weights (seminal vesicles and coagulating glands) were not affected by EGEE. Our current study has also demonstrated that testis weight was decreased by EGEE. EGEE has been associated with testicular toxicity in laboratory animals in several studies, one of which dates back to 1942 (6). In addition, we showed for the first time that fertility was dramatically suppressed and sperm head abnormalities increased in EGEE-treated male mice. The drinking water consumption data and mean body weight data indicated that the male mice which had 1% EGEE in the water received about 1500 mg EGEE/kg per day and the mice receiving 2% EGEE in the water received about 2600 mg EGEE/kg per day. Water consumption was reduced in the 2% dose group mice without any significant loss in body weight.

The effects of EGEE on female mice might be related to embryotoxicity, fetotoxicity or to a direct antifertility effect of EGEE. The teratogenicity of EGEE has been demonstrated in rats and rabbits by inhalation exposure (7) and rats after dermal exposure (3). However, Andrew et al. (7) detected no adverse effect on fertility of female rats exposed to EGEE by inhalation for 3 weeks.

There was evidence of a drop in offspring survival, and a decrease in fertile matings was detected in the EGEE-treated females. The majority of studies on the

toxicity of glycol ethers in females published to date have involved gestational exposure to the compounds (6). Those studies clearly demonstrated the teratogenic potential of these compounds. In our investigations, the female mice that received 2% EGEE never delivered a single offspring, alive or dead. The females receiving 1% had fewer live pups per litter. The teratogenicity of EGEE may be responsible for the decreased pup survival, but the decreased fertility in the female mice could be related to gonadal, endocrine or reproductive tract toxicity. These aspects of EGEE toxicity will be addressed in later studies.

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