Challenges in the Toxicological(Mutagenic and Teratogenic)/Environemental Methods under the GLP System

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GLP regulations were initially "promulgated to address assuring the validity of data in the wake of investigations by EPA and FDA during the mid –1970's which revealed that some studies submitted to the agencies had not been conducted in accordance with acceptable laboratory practices." [1]

In the early 1970s, results of an investigation by the FDA in about 40 laboratories revealed many cases of badly managed studies, poor training of personnel and some cases of deliberate fraud. The general findings were that there were poorly trained study directors and study personnel, poorly designed protocols, protocols not followed, procedures not conducted as described, raw data badly collected, data not correctly identified, data without traceability, data not verified and approved by responsible persons, lack of standardised procedures, poor animal husbandry, inadequate characterisation of test items and test systems, inadequate resources, equipment poorly calibrated or otherwise qualified, reports not sufficiently verified, not an accurate account of the actual study, not a proper reflection of raw data and inadequate archiving of data. These problems are not just past history, since they resurface time and time again, even in quite recent times as the experience of GLP inspectors shows [1]. The GLPs specify minimum practices and procedures in order to ensure the quality and integrity of data submitted in accordance with a regulatory requirement

The idea behind GLPis that it will help scientists to obtain results which are reliable, reproducible, auditable and recognised by scientists worldwide. GLP requires the education and training of personnel, the maintenance and calibration of instruments, standard operating procedures, test system characterisation, test item identification, characterisation and accountability, study planning and any intentional or unintentional changes to the study, indelible recording of data confirmed by dating and initialling, complete reporting of all data and not a selection fitting the hypothesis.

GLP is therefore defined as a managerial tool and a quality system concerned with the organisational process and the conditions under which non-clinical safety studies are planned, performed, monitored, recorded, archived and reported [2]. It does not rule the scientific aspects of the study, but merely ensures the reconsructability and the reliability of the study.

I shall illustrate my talk with some studies of male-mediated cogenital malformations in mice after treatment with various chemicals, but first I shall provide some background information

Current guidelines for regulatory testing require that only the female is tested for teratogenic effects. However, since the male contributes half of the genetic information of the genome to developing offspring, then males could also be examined for induced "teratogenic" effects (congenital malformations). Transplacental carcinogenesis is recognised in the female, but carcinogenesis mediated through the male germ cells is not so well appreciated and understood. Nevertheless, in recent years, the public has become more aware that exposure of males to certain agents can adversely affect their offspring; for example, smoking fathers appear to give rise to tumours in the F_1 generation [3-5]. Also, Lefebvre *et al.* [6] have shown that the paternally transmitted and paternally imprinted gene, MEST, is involved in normal maternal behaviour. MEST-deficient females show abnormal behaviour and intrauterine and postnatal growth retardation of progeny. This is even more evidence of how important the male is to the successful development of the future generation.

In the case of low dose chronic radiation exposures as at the Sellafield nuclear plant, there was thought to be an association between exposure of men working at Sellafield and leukaemia in their children [7,8]. This was subsequently criticised [9] and after exhaustive investigations, it appeared that paternal radiation exposure was unlikely to be responsible for excesses of childhood leukaemia or non-Hodgkin lymphoma in Britain [10]. However, Savitz [11] has reported an increased incidence of miscarriages after potential

exposure to a variety of agents. Radiation exposure at Chernobyl has also been found to have induced heritable mutations in the male germ line [12].

Congenital malformations and tumours can be studied after exposure of male rodents in an extended dominant-lethal assay where untreated females mated to treated males are examined the day before term, as opposed to mid-term in the conventional study [13]. At this stage, congenital malformations, such as hydrocephaly, exencephaly, cleft palate, open eye, runts (dwarfs), oedema, anasarca and gastrochisis can be determined. Some of these abnormalities have similar manifestations in humans. The foetuses can also be examined for skeletal malformations by using alizarin staining. If the F_0 treated and control males are mated with more than one female, then in the F_1 generation, litters of the extra female(s) can be examined for the same effects in live-born offspring, confirming the original observation. Litters can also be allowed to develop to adulthood where tumours can be observed and karyotype analysis can be performed on foetuses and adult offspring to determine if induced genetic damage can be transmitted.

Application of Study Design to three paternal exposures

By using this type of study design, we have examined the following compounds: cyclophosphamide, 1,3-butadiene and urethane, using chronic and acute exposure. A summary of the findings is shown in Table 1 [references 14-20]. Results from these chemicals follow:

Table 1. Responses in different studies in rats and mice

		Treatment			Endpoints					
							F_1 karyotype		F_1 tumours	
Compound	Species	Acute	Sub-chronic	Chronic	F_0 dominant lethal mutation	F_1 Congenital malform-ations	Foetus	Adult	Adult	Refs
Cyclophos-phamide	Mouse[a]	T			+	+	–	ND	ND	14
	Rat[b]			T	+	+	+	+	±	15,16
1,3-butadiene	Mouse[a]	T			+	–	–	ND	–	17
	Mouse[a]		T		+	+/–	–	ND	–	18,19
	Rat[b]		T		–	ND	ND	ND	ND	17
Urethane	Mouse[a]	T			–	–	–	–	+[c]	20
			T		–	–	–	–	–	20

[a]CD-1 mice; [b]Sprague-Dawley rats; [c]Males only.
*+=statistically significant increase above untreated controls; – = no statistically significant increase; ± = equivocal response; +/– = one study positive, one negative; ND = not done.

Cyclophosphamide was positive in the rat after chronic gavage exposure for 33 weeks, for endpoints of dominant lethal mutation plateauing at 75% after week 7 [15,16], congenital malformations (Table 2) and tumours (Table 3). F_1 karyotype analysis both in the foetus (Table 4) and adults was carried out where chromosome abnormalities were found in all cells of 2 of the adults, confirming transmission of induced damage through the male germ line [16]. Such effects with cyclophosphamide have also been shown by other workers [21] and led to the belief that chronic exposure might be a more realistic model than acute exposure, since in the workplace and environmentally, man is chronically exposed.

Table 2. Characterisation of Sprague-Dawley rat foetal abnormalities after cyclophosphamide and allyl alcohol treatment of F_0 males

Abnormality	Control	Allyl alcohol[a]	Cyclophosphamide[b]
Anasarca		1	13 (7)[c]
Anasarca + craniofacial abnormality			3 (2)
Anasarca + skeletal abnormality			4 (2)
Exencephaly		1	
Hydrocephaly			6
Craniofacial abnormality			6
Craniofacial and skeletal abnormality		1	2
Anaemia			4
Gastroschisis			2 (1)
Abnormal placenta			1
Growth retarded foetuses (Runts)	13	13	61
Total	13	16	102

[a]25 mg/kg body weight; [b]3.5 mg/kg body weight for 4 weeks and 5.1 mg/kg body weight from weeks 5-33 subsequently. [c]Numbers in parentheses indicate dead foetuses. Allyl alcohol is a metabolite of cyclophosphamide.

Table 3. Tumours and hydronephrosis identified macroscopically at post-mortem in female offspring from cyclophosphamide-treated and control male Sprague-Dawley rats*

Abnormalities identified macroscopically at post mortem	Histological findings	Paternal treatment	Age at post mortem (weeks) Upto 53	54-66	67-79	80-91	92-104	Total
Hydronephrosis	–	CP	2#	2c##	1d#	3###	2e##	10
		Control	1	0	1	1b#	1a	4
Liver tumour	Fibrosarcoma	CP	0	0	0	0	0	0
		Control	0	0	0	0	1a	0
Lung tumour	Fibrosarcoma	CP	0	0	0	0	0	0
		Control	0	0	0	0	1a	1
Pituitary tumour	Adenoma	CP	0	0	0	0	2e#	2
		Control	0	1	0	1b	1a	3
Lymph node tumour	Adenofibroma	CP	0	0	0	0	0	0
		Control	0	0	1#	0	0	1
Vaginal tumour	Fibrosarcoma	CP	0	1	0	0	0	1
		Control	0	0	0	1	0	1
Ovarian tumour	Fibrosarcoma	CP	0	0	0	0	0	0
		Control	0	0	0	0	1a	1
Mammary tumour	Total (including tumours not examined)	CP	1	5	5	4	4	19
		Control	1	2	8	8	4	23
	Adenofibroma	CP	–	1	1	1	–	3
		Control	–	–	–	1	2	3
Uterine tumour	Total (including tumours not examined)	CP	0	1	1d	1	1#	4f
		Control	0	0	0	0	0	0
	Sarcoma	CP	0	1c	–	0	–	1
		Control	0	0	0	0	0	0
	Carcinoma	CP	0	0	–	1#	–	1
		Control	0	0	0	0	0	0
Total number of female offspring		CP	10	14	8	9	6	47
		Control	9	19	18	14	7	67

*Some tumours were examined histologically, and the findings are shown in the table. #Indicates each animal from which a karyotype was analysed (note that some animals had more than one macroscopic abnormality; see a–e). [a]All these abnormalities found in one animal; similarly, [b,c,d,e]; CP = cyclophosphamide; – = not examined histologically, [f]=borderline significance (p=0.051) by comparison with controls; doses as in Table 2.

Table 4. Results of analysis of abnormal Sprague-Dawley rat foetus karyotypes after cyclophosphamide and allyl alcohol treatment of F_0 males

Foetus No.	Abnormality	Centromeres (No.)	Karyotype abnormality
AA 42	Runt	43 (46)[a]	Tris. + 3 Fragments
AA 48	Anasarca/Runt	43	Tris. Ch. not identified
AA 94	Craniofacial	43	Tris. Ch. not identified
CP 29	Runt	42	Trans. intra Ch. 1
CP 31	Runt	42	Trans. Ch. 6 → Ch. 2
CP 58	Runt	42	Trans. Chs. not identified
CP 60	Runt	41	Monos. + Trans. Ch. 2 → Ch. 3
CP 64	Runt	42	Trans. Acrocentric Ch. → Ch. 2
CP 82	Runt	41	Monos. + Trans. Ch. 4/5 → Ch. 3
CP 90	Runt	42	Trans. Ch. 1 → Ch. 2
CP 91	Runt	42	Trans. Ch. 1 → Ch. 2
CP 106	Runt	42	Deletion Ch. 1
CP 51	Anaemic/Runt	42	Trans. Ch. 3 → Ch. 2
CP 70	Anaemic	41	Monos. Ch. 2 + Trans. Ch. 17/2 → Ch. 1
CP 120	Anaemic	41	Monos.
CP 63	Anasarca/Runt	42	Trans. Ch. 3 → Ch. 13
CP 100	Anasarca	42	Trans. Ch. 5 → Ch. 1
CP 59	Craniofacial	43	Tris. Ch. 4/5
CP 110	Craniofacial	42	Trans. Ch. 10 → Ch. 2
CP 52	Hydroceph./Runt	42	Trans. Chs. not identified
CP 101	Skeletal	41	Monos. + Trans. Chs. not identified
CP 66	Abnormal placenta	42	Trans. Ch. 4/5 → Ch. 2

Ch. = Chromosome; Trans. = Translocation; Monos. = Monosomy; Tris. = Trisomy; [a]3 small fragments were present in every metaphase and may have been centromeric; CP = cyclophosphamide; AA = allyl alcohol; doses as in Table 2.

In mice, 1,3-butadiene was positive for endpoints of dominant lethal mutation and congenital malformations after 10 weeks' exposure (Table 5), even when compared to the historical control congenital malformation data (Table 6). There were significant effects in one study [18] and not in another [19] with no increase in tumours after sub-chronic inhalation exposure [18] (Table 7). In the rat no dominant lethality was observed after 10 weeks' exposure and there were no increases in congenital malformations in mice after 4 weeks' exposure [17].

However, for urethane there were negative results in mice for dominant lethality and congenital malformations after sub-chronic exposure in the drinking water (Table 8), although there was an increase in tumours in males after acute intraperitoneal (ip) treatment [20] (Table 9). A Japanese study in ICR mice after acute ip treatment, also obtained negative dominant lethal mutation results, confirming results by other workers, but showed an increase in congenital malformations, tumours in the F_1 generation and transmitted tumours in the F_2 and F_3 generation [22].

Table 5. Effect of 1,3-butadiene on reproductive outcome in CD–1 mice after subchronic (6 h/day, 5 days/week, 10 weeks) exposure of males

Treatment	No. of males	Males mated to at least 1 female		No. of females[a]	Pregnant females		No. of pregnant females used in assay for dominant lethal mutation[b]
		n	%		*n*	%	
Control	25	23	92	50	41	82	23
12.5 ppm	25	25	100	50	45	90	24
1250 ppm	48[c]	43	90	96	74	77	38

	Implantations		Early deaths		Late deaths		Late deaths including dead foetuses		Abnormal foetuses	
	n	Mean ± S.D.	*n*	Mean ± S.D.	*n*	Mean[d] ± S.D.	*n*	Mean[d] ± S.D.	*n*	Mean[d] ± S.D.
Control	278	12.09±1.28	13	0.050±0.059	0		2	0.007±0.022	0	
12.5 ppm	306	12.75±2.51	16	0.053±0.058	7	0.023**±0.038	8	0.026*±0.042	7[e]	0.024*±0.062
1250 ppm	406	10.68**±3.10	87	0.204***±0.161	6	0.014***±0.032	7	0.016*±0.034	3[f]	0.011**±0.044

Significantly different from control at $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ (by analysis of variance and least significance test on arc-sine transformed data).
[a]Each male housed with two females for up to 1 week; [b]Not more than one for each male; the other females were allowed to litter; [c]2/50 males died after treatment (one at week 5, one at week 1); [d]Per implantation per pregnancy; [e]Four exencephalies (three in one litter), two runts (70% and 60% of mean body weight of others in litter, total litter sizes, 7 and 9, respectively), one with blood in amniotic sac but no obvious gross malformation (significance of difference not altered if this foetus is excluded); [f]One hydrocephaly, two runts (71% and 75% of mean body weight of others in litter; total litter sizes, 2 and 11, respectively).

Table 6. Results of assays for dominant lethal mutations and congenital malformations in CD–1 mice in previous studies (i.e. historical control data)

Study	No. of	No. of live foetuses		No. of abnormal foetuses	No. of late deaths	No. of early deaths
Jenkinson *et al.* (1987) Experiment 1	720	671	8	(1 Exencephaly, 1 open eye, 6 runts)	7	34
Jenkinson *et al.* (1987) Experiment 2	1212	1125	6	(1 Exencephaly, 1 open eye, 4 runts)	19	62
Anderson *et al.* (1987)	–	680	2	(1 Exencephaly, 1 open eye)	–	–
Anderson *et al.* (unpublished) Experiment 1	254	234	5	(1 Hydrocephaly, 4 runts)	3	12
Anderson *et al.* (unpublished) Experiment 2	279	259	1	(1 Bent hind limb)	3	16
Totals	2465	2969	22		32	124
Percentage of number of implants					1.30%	5.03%[a]
Percentage of number of live foetuses			0.74%			

[a]A similar value (5.37%) for the percentage of early deaths in the CD–1 mouse strain was obtained in the 1970s and 1980s in over 700 foetuses [23].

Table 8. The effect of urethane in an extended dominant lethal assay in CD–1 mice after acute (intraperitoneal) or sub-chronic (drinking water) exposure of males

Treatment	Number of pregnant females[a]	Implantations (per pregnant female)[b,c]	Early deaths[b,d]	Late deaths[b,d]	Abnormal foetuses[b,d,e]
Acute					
Control	24	11.58±2.39	0.069±0.10	0.011±0.03	0.003±0.01
1.25 g/kg bwt	18	12.06±3.04	0.018±0.03	0.008±0.02	0.014±0.06
1.75 g/kg bwt	15	11.80±2.54	0.049±0.08	0.012±0.03	0.005±0.02
Sub-chronic					
Control	20	12.50±3.38	0.093±0.23	0.012±0.03	0.028±0.05
1.25 mg/ml for 10 weeks	23	12.91±1.73	0.014±0.03	0.023±0.04	0.006±0.02
3.75 mg/ml for 9 weeks	20	11.25±2.34	0.028±0.05	0.015±0.04	0

[a]Approximately half the pregnant femaleswere allowed to litter; [b]Mean and standard deviation shown; [c]Tested using two-sample t-test (no significant differences found); [d]Data expressed per implantation per female. Transformed data [24] tested using two-sample t-test (no significant differences found); [e]See text for description of abnormal foetuses.

It is assumed that such effects as shown above could only be produced as a result of alterations induced in the exposed males' germ cells, because of their heritability through the generations.

Table 7. Summary of tumour incidence for F_1 adult offspring from CD-1 male mice treated subchronically with butadiene

Tumour site	Sex	F_1 animals			Treatment group					
		Control			12.5 ppm Butadiene			1250 ppm Butadiene		
		Age at necropsy (weeks)			Age at necropsy (weeks)			Age at necropsy (weeks)		
		No. of animals	<74[a]	74–75[b]	No. of animals	<74[a]	74–75[b]	No. of animals	<74[a]	74–75[b]
Liver	Male	11	1 (71)	10	22	2 (66,70)	20[d]	25	3 (49,54,1)	22[h]
	Female	1	1 (54)	0	1	0	1	1	0	
Kidney	Male	0	0	0	0	0	0	1	1 (49)	0
	Female	1	1 (49)	0	0	0	0	1	1 (66)	0
Lung	Male	1	0	1	2	0	2	2	1 (69)	1[f]
	Female	0	0	0	0	0	0	4	2 (54,71)	1
Limb	Male	0	0	0	0	0	0	2	2 (45,52)	0
	Female	2	2 (45,52)	0	0	0	0	0	0	0
Urinogenital	Male	0	0	0	0	0	0	1	1 (48)	0
	Female	1	1 (48)	0	0	0	0	0	0	0
Stomach	Male	0	0	0	1	0	1[e]	0	0	0
	Female	0	0	0	0	0	0	0	0	0
Pancreas	Male	0	0	0	1	0	1[f]	0	0	0
	Female	0	0	0	0	0	0	0	0	0
Abdominal	Male	0	0	0	1	1 (51)	0	0	0	0
	Female	0	0	0	0	0	0	1	1 (70)	0
Total with at least one tumour	Male	12	1	11	25[g]	3	22	30[k]	8	22
	Female	5[c]	5	0	1	0	1	7[i]	4	3
No tumours	Male	85	12	73	72[g]	19	63	68[k]	4	64
	Female	114[c]	14	100	106	26	80	93[i]	21	72
Total	Male	97	13	84	102[g]	22	85	100[k]	12	86
	Female	121[c]	19	100	107	26	81	101[j]	25	75

[a]Number in parentheses indicate age (weeks) at which animal was humanely killed or died due to sickness.
[b]All surviving animals were humanely killed for necropsy at 74–75 weeks.
[c]No data available for two F_1 females.
[d]Also stomach and pancreas; [e]Also liver and pancreas; [f]Also liver and stomach; [g]No data available for five F_1 males; [h]Also lung; [i]Also liver;
[j]No data available for one F_1 female; [k]No data available for two F_1 males.

Since tumours can be manifest without dominant lethal mutations as is the case for urethane (see Tables 1 and 9) [20], the different endpoints may be independent genetic (germ cell transmissible) events and might be animal species and/or strain dependent (see mice versus rats after 1,3-butadiene exposure in Table 1). The question of acute versus chronic exposure might also be agent/compound dependent (Table 1).

The exact time of mating, within the week after treatment, and local husbandry conditions can have an effect on observed responses. In order to obtain sufficient numbers of offspring for analysis, there is a delicate balance between death through dominant lethality and survival of normal and malformed offspring creating a "window" for detection of effects. As with any toxicological model, careful control of parameters is required. However, it is a useful model for examining inherited congenital malformations and tumours which can be attributed to exposure of the male and could be useful for predicting possible effects in man.

In the human situation e.g. after chemotherapy, males can be advised not to indulge in sexual practices for a few months, to allow progression of newly formed germ cells through the next spermatogenic cycle, and often males do not feel well enough to mate. Thus, the animal models might not exactly reflect the human situation because animals are constantly mating.

However, there can also be difficulties in detecting reproductive effects directly in humans e.g. when interviewing for reproductive outcome. This can be illustrated by reproductive studies with vinyl chloride. Personal interviews and/or questionnaires are a primary source of data for monitoring programmes. In gathering information covering reproductive events, studies based on husbands' indirect reports yielded considerably lower figures for pregnancy loss [25] then those based on interviews with wives [26]. Individuals clearly have a much better recall for events in their own lives, and the circumstances of pregnancy are far more significant for a woman than a man. Therefore gathering information directly from the wives of employees would be a valuable technique in industrial male reproductive monitoring programmes. Therefore, even human models are not perfect and animals models can provide useful information for man. Due to difficulties in conducting controlled reproductive studies in humans, it is important to use model systems in rodents to try to understand how paternal exposure could result in congenital abnormalities in offspring of man and/or produce a predisposition to cancer. Further work is desirable using these model systems with other chemicals to try to understand how predictive they are for man.

Having completed this series of studies, we were contacted by the US Environmental Protection Agency (EPA). EPA were very interested in the data that we had generated on 1,3-butadiene, the studies having been funded by the EU and the Rubber Manufacturers' Association. Butadiene is produced in large quantities for use in the manufacture of synthetic rubber. It is also an environmental pollutant as it is present in car exhaust fumes. Permission was sought from both organisations to be allowed to provide the EPA with the raw data from these studies which had been conducted under conditions of GLP. Instead of using a cut-off of 75% of mean litter weight for runts, the EPA wished to investigate our data using weight of pups as a continuous variable to determine if this was a more sensitive parameter for determining congenital malformations. This was because we were detecting such malformations at 12.5 ppm and this was, at the time of the study, around the maximal exposure limit as an 8-hr time weighted average [HSE, 27]. The US Occupational Safety and Health Administration [28] proposed lowering the permissible occupational exposure limit to 2ppm, so our data at that time and subsequent studies helped in determining if it was appropriate to lower the limit. Apparently, it made little difference whether continuous or discrete variables were used, but the data would not have been suitable for EPA use if GLP studies had not been conducted.

Table 9. The effect of urethane on tumour incidence in the offspring of CD–1 mice after acute (intraperitoneal) exposure of males (number of F_1 animals shown)

Paternal treatment (acute):		Control			1.75 g/kg bwt		
Tumour site	Sex	Total	45–82 weeks old	83–84 weeks old	Total	45–82 weeks old	83–84 weeks old
Kidney	M	1	1	0	0	0	0
	F	1	0	1	1	1	0
Limb	M	1	1	0	2	2	0
	F	5	4	1	1	1	0
Liver	M	10	1	9	23*	6	17
	F	1	0	1	0	0	0
Lung	M	5	1	4	6	2	4
	F	1	1	0	2	1	1
Pancreas	M	0	0	0	0	0	0
	F	0	0	0	2	0	2
Thymus	M	0	0	0	1	0	1
	F	0	0	0	0	0	0
Urinogenital	M	0	0	0	0	0	0
	F	1	0	1	0	0	0
Uterus/ovaries	F	4	1	3	4	4	0
Total with at least one tumour	M	17	4	13	27	8	19
	F	13	6	7	10	7	3
Total with no tumours	M	81	18	63	83	27	56
	F	86	23	63	87	12	75
Total no. of F_1 animals	M	99	22	76	111	35	75
	F	100	29	70	97	19	78

*Statistically significant from controls p=0.026.

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