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DIFFERING RESPONSE OF THE GLUTATHIONE SYSTEM TO FASTING IN NEONATAL AND ADULT GUINEA PIGS

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Abstract—Adult, term neonatal and 3 day preterm neonatal guinea pigs were fasted for 48 hr, and the glutathione concentrations of the liver and lung assessed. In adult animals, glutathione concentration decreased by 43% in the liver and 29% in the lung with respect to fed controls. The decrease in liver glutathione was associated with a 75% reduction in the hepatic activity of τ-glutamyltranspeptidase (τGGT). Conversely, both liver and lung glutathione levels in preterm pups remained unchanged following 48 hr food restriction. Likewise, hepatic τGGT, glutathione reductase (GRed) and glutathione peroxidase (GPx) activities were unchanged by fasting in preterm pups. Fasting increased pulmonary GPx activity by 27% in these pups. In fasted, term animals, substantial increases in both lung (65%) and liver (80%) glutathione concentrations were observed, with concomitant increases in GPx and GRed activities. Hepatic τGGT activity was significantly reduced (57%) in term pups. These results may suggest that the neonatal guinea pig can maintain tissue glutathione status during periods of nutrient stress, through an increased capacity for recycling oxidized glutathione and a decrease in turnover of the tripeptide. Guinea pig neonates are therefore able to resist starvation-induced decreases in tissue glutathione levels seen in adult rodents. If this is a general neonatal response it may have important clinical implications in the treatment of preterm babies.

Glutathione (reduced form GSH†) is a tripeptide involved in a wide range of metabolic functions, primarily aimed at providing protection against toxic compounds and free radical species [1]. In this respect, GSH is believed to play a primary role in the protection of the lung from oxidative injury [1]. Although, under appropriate conditions, oxidized glutathione (GSSG) can be reduced back to GSH, *de novo* GSH synthesis is a prerequisite to maintain GSH concentrations during oxidative stress. GSH synthesis and storage is believed to occur primarily in the liver, prior to its release into the circulation and breakdown into precursors by kidney τ-glutamyl transpeptidase (τ-GGT). The dipeptide and amino acid products of GSH breakdown are taken up and used for resynthesis of GSH by extra-hepatic tissues [2]. GSH synthesis is dependent upon an adequate supply of cysteine [1, 3]. Cysteine is derived either directly from dietary protein, or indirectly from endogenous methionine. In adult rodents, fasting for periods of 12–48 hr depletes hepatic GSH by up to 50% [4–9]. It therefore follows that hyperoxia-induced lung injury seen in premature babies undergoing ventilator therapy may be exacerbated by their poor nutrient and, hence, compromised GSH status. Indeed, studies in adult rats and mice have indicated that fasting or chemical-mediated GSH depletion increases oxygen free radical-mediated lung injury [4, 5, 10–13]. Our own studies, employing a guinea pig model of prematurity, have not however supported a role for GSH in the increased lung injury seen in fasted animals exposed to hyperoxia [14].

In this paper we demonstrate that the responses of pulmonary and hepatic GSH concentrations, and associated enzyme activities, to starvation differ markedly in preterm and term neonates compared to adult guinea pigs.

MATERIALS AND METHODS

Chemicals. All chemicals and reagents were purchased from the Sigma Chemical Co. (Poole, U.K.) or BDH (Poole, U.K.) unless stated otherwise in the text.

Animals. Virgin female Dunkin–Hartley guinea pigs (550 g) bred in the Rayne Institute animal facility were caged in pairs in a temperature-controlled room (22–24°) on a 12 hr light : 12 hr dark cycle. Animals had free access to water. Guinea pig chow (SDS, Witham, U.K.), carrots and cabbage were provided. The chow was by weight 0.29% cysteine and 0.38% methionine. Adult guinea pigs in the colony were also administered 20 mg vitamin C in the drinking water each week. Timed pregnancies were established as described previously [15]. Pregnancies were allowed to progress to either full term (day 68), with a normal vaginal delivery, or day 65 of gestation, when pups were delivered by caesarian section [15]. All pups were delivered within 5 min of the initial anaesthetic administration. The dams were killed by exsanguination.

Term and preterm pups, or adult animals were housed in 25 L capacity purpose-built plastic boxes

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† Abbreviations: GSH, glutathione (reduced); GSSG, glutathione (oxidized); GPx, glutathione peroxidase; GRed, glutathione reductase; τGGT, τ-glutamyl transpeptidase.

with wire mesh bottoms over sterile sawdust. Cages were washed and disinfected daily. Animals were allocated to a fed or fasted treatment. Fed adults were given access to standard guinea pig chow and straw. Fasted animals were housed without straw or solid food. Fed neonates were housed with lactating surrogate mothers, with access also to guinea pig chow and straw. Fasted neonates were housed with non-lactating adult females, with no chow or straw in the cage. After 48 hr, sodium pentobarbital anaesthesia was administered. All animals were killed between 0730 and 0830 hr. The lungs and livers of the animals were excised, rinsed in ice-cold 0.9% saline, blotted dry and weighed. A portion of each tissue (200 mg) was removed for GSH analysis, and the remaining tissue frozen in liquid nitrogen, and then stored at −80° for up to 2 weeks, for subsequent analysis of antioxidant enzymes.

A total of nine adults (four fed, five fasted), 16 term pups (seven fed, nine fasted) and 15 preterm pups (seven fed, eight fasted) were used.

Determination of glutathione. Glutathione was determined by the method of Tietze [16]. Fresh lung and liver samples (0.2 g) were homogenized in 3 mL 0.2 N perchloric acid and centrifuged at 2000 rpm for 10 min. GSSG concentrations were below the detection limits of the assay (0.1 μmol/g tissue), and did not rise above this value during fasting.

Determination of enzyme activities. Lung and liver samples (400 mg) were prepared as described previously [17]. Following preparation, 80–90% of activity in the homogenate is typically recovered (Kelly *et al.*, unpublished observations).

Glutathione peroxidase (GPx) activity was assayed by the method of Beutler [18]. Glutathione reductase (GRed) was assayed using a Biotek microplate reader. To each well of a microtitre plate homogenate (30 μL) and working reagent (200 μL) were added. Working reagent contained 0.1 M sodium phosphate buffer pH 7.6, 0.1 mM NADPH, 0.5 mM EDTA and 1.0 mM GSSG. A_{340} was followed over 1 min at 30°. τGGT was assayed using a kit obtained from Sigma, based upon the assay of Orlowski and Meister [19]. All enzyme activities were expressed per milligram protein. Protein was assayed by the method of Smith *et al.* [20].

Statistical analysis. Data were analysed using a two-way analysis of variance (ANOVA) for an unbalanced design. Where significant interactions were indicated, statistical probability was determined using Student's *t*-test. A probability value of less than 5% was taken as significant.

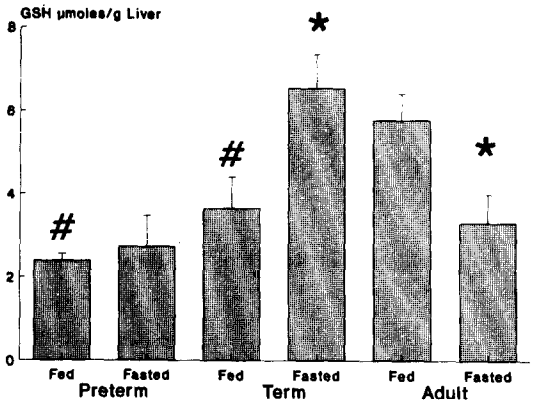

Fig. 1. Total glutathione concentrations in the livers of fed and starved guinea pigs of different ages. Adult, term and preterm guinea pigs were treated as detailed in the text. Values are means ± SEM. # Indicates liver glutathione was significantly influenced by age ($F = 8.24$, 2,37, $P < 0.001$). * Indicates fasting significantly altered liver glutathione in an age-dependent manner ($F = 6.51$, 2,37, $P < 0.005$).

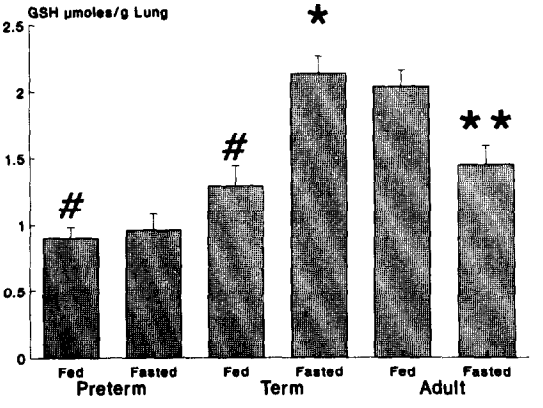

Fig. 2. Total glutathione concentrations in the lungs of fed and starved guinea pigs of different ages. Adult, term and preterm guinea pigs were treated as detailed in the text. Values are means ± SEM. # Indicates lung glutathione was significantly influenced by age ($F = 28.32$, 2,37, $P < 0.001$). *, ** Indicates fasting significantly altered lung glutathione in an age-dependent manner ($F = 14.73$, 2,37, $P < 0.001$).

RESULTS

Tissue and glutathione concentrations

Body weight changes of the animals were followed over the 48 hr of the study. Fasted preterm pups did not lose significantly more weight than their fed littermates (10% fasted, 16% fed). Term pups fasted for 48 hr lost 19% of initial body weight, whilst fed pups lost 5% of birthweight. Fasted adults lost 9% of initial body weight, whilst the weight of fed adults did not alter over the 48 hr of the study.

The concentration of glutathione in the liver (Fig. 1) increased significantly during development from preterm to adult ($P < 0.001$, $F = 8.24$, 2,37). A similar trend was also observed in the lung (Fig. 2) ($P < 0.001$, $F = 28.32$, 2,37). Food restriction for 48 hr led to a 43% decrease in hepatic glutathione concentrations in adult animals (Fig. 1). In preterm neonates, fasting did not alter hepatic glutathione status, while in term pups, liver GSH concentration increased 80% ($P < 0.05$). In adults, lung glutathione concentration decreased by 29% following a 48 hr

Table 1. The effects of fasting on tissue protein concentrations in the lungs and livers of preterm, term and adult guinea pigs

	Protein (mg/g tissue) Lung	Liver
Preterm		
fed	154.0 ± 17.7	202.5 ± 27.0
fasted	184.0 ± 13.4	161.0 ± 13.8
Term		
fed	90.4 ± 9.3	231.0 ± 5.2
fasted	167.2 ± 23.1	213.0 ± 19.7
Adult		
fed	83.5 ± 19.5	225.0 ± 17.7
fasted	205.6 ± 16.4*	258.2 ± 27.1

Adult, term and preterm guinea pigs were treated as detailed in the text.

Values are means ± SEM.

* Indicates significant difference between fed and fasted animals of the same age $P < 0.05$.

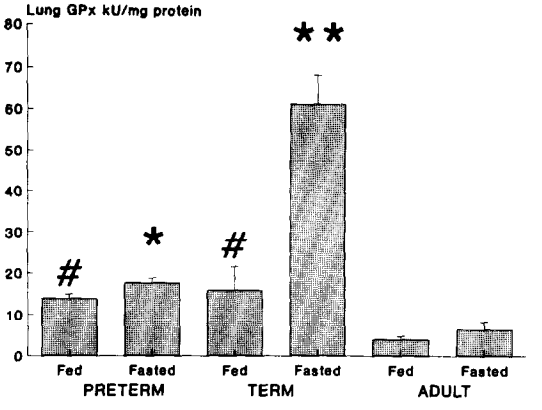

Fig. 4. GPx activity in the lungs of fed and starved guinea pigs of different ages. Adult, term and preterm guinea pigs were treated as detailed in the text. Values are means ± SEM. Lung GPx was significantly influenced by age ($F = 22.64$, 2,21, $P < 0.001$). # Indicates significantly different to adult ($P < 0.05$). *, ** Indicate fasting significantly altered lung GPx activity ($F = 9.37$, 2,21, $P < 0.01$).

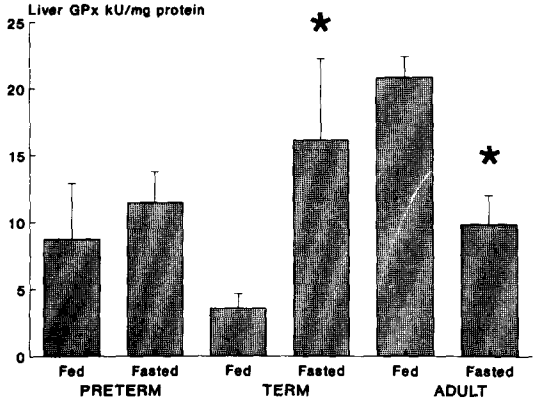

Fig. 3. Liver GPx activity in fed and starved guinea pigs of different ages. Adult, term and preterm guinea pigs were treated as detailed in the text. Values are means ± SEM. * Indicates fasting significantly altered liver GPx activity in an age-dependent manner ($F = 3.86$, 2,29, $P < 0.05$).

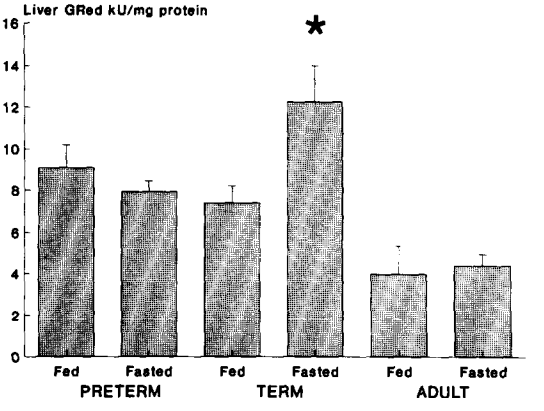

Fig. 5. GRed activity in the livers of fed and starved guinea pigs of different ages. Adult, term and preterm guinea pigs were treated as detailed in the text. Values are means ± SEM. * Indicates fasting significantly altered liver GRed activity ($F = 10.36$, 2,21, $P < 0.005$).

fast (Fig. 2). As seen in the liver, the pulmonary glutathione status of the preterm pups was unaltered by fasting. Fasted, term neonates exhibited a 65% increase in pulmonary glutathione, relative to fed controls.

Hepatic and pulmonary glutathione-related enzyme activities

All enzyme activities were expressed per milligram protein. Protein concentrations in the lung and liver were determined and are shown in Table 1. No significant effects of fasting were observed, except in the lungs of fasted adults where there was an increase of 146%.

Hepatic GPx exhibited age-dependent responses to a 48 hr fast (Fig. 3). In adults, a significant decrease in GPx activity was observed. In term neonates, 48 hr fasting led to a 4-fold increase in hepatic GPx activity, while in preterm pups, no change in GPx activity was observed. Pulmonary GPx activity (Fig. 4) was unchanged in adults following fasting, but in both groups of neonates significant increases in activity were observed following fasting. In preterms this increase was small (27%), while in term pups a 350% increase was observed.

Hepatic GRed activity was significantly lower in adults than in neonates (Fig. 5) ($P < 0.001$, $F = 10.50$, 2,29). No significant changes in hepatic or pulmonary GRed activity were observed in adults

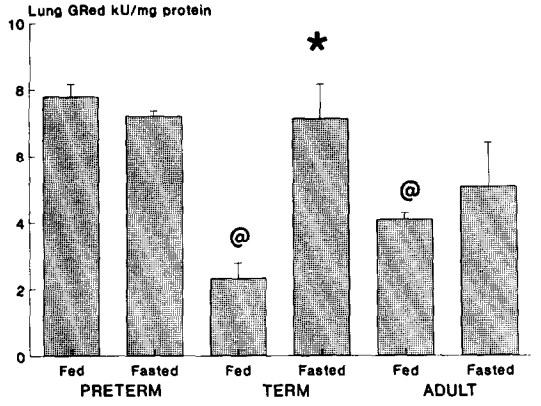

Fig. 6. GRed activity in the lungs of fed and starved guinea pigs of different ages. Adult, term and preterm guinea pigs were treated as detailed in the text. Values are means ± SEM. Lung GRed activity was significantly influenced by age ($F = 10.50$, 2,29 $P < 0.001$). @ Indicates significantly different to preterm ($P < 0.05$). * Indicates fasting significantly altered lung GRed activity in an age-dependent manner ($F = 3.46$, 2,29, $P < 0.05$).

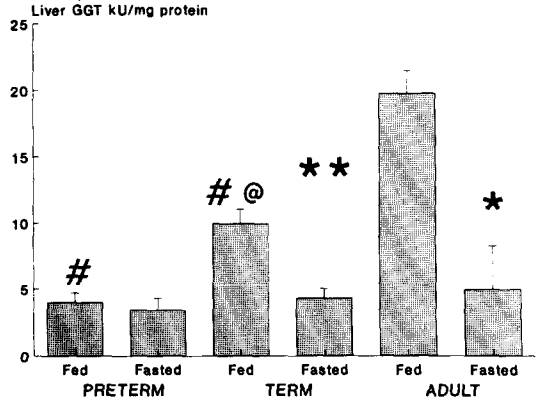

Fig. 7. GGT activity in the livers of fed and starved guinea pigs of different ages. Adult, term and preterm guinea pigs were treated as detailed in the text. Values are means ± SEM. Liver τGGT activity was significantly influenced by age ($F = 107.36$, 2,29 $P < 0.001$). # Indicates significantly different to adult ($P < 0.05$). @ Indicates significantly different to preterm ($P < 0.05$). *, ** Indicate fasting significantly altered liver τGGT activity. ($F = 32.96$, 1,29, $P < 0.05$).

or preterm pups following a 48 hr fast (Fig. 6). In term neonates, GRed activity in both tissues increased significantly in response to food restriction. This increase was most marked in the lung (300%).

Hepatic τGGT activity (Fig. 7) increased with age ($P < 0.001$, $F = 107.36$, 2,29). Activity of this enzyme was unaltered by fasting in preterm neonates, but was decreased by 57% and 75% in term pups and adults, respectively.

DISCUSSION

The present study has demonstrated pronounced differences in the response of adult and neonatal guinea pigs to fasting. In adult animals, fasting produced a decrease in glutathione concentrations and no apparent alterations in GRed activity in either the liver or lung (although in the lung the increase in protein concentration on fasting may lead to an underestimation of GRed activity). This observation supports previous findings in guinea pigs [8] and in other rodents [6, 11, 21–23]. Contrary to these findings, we have shown that fasting does not alter tissue glutathione concentrations in the preterm guinea pig, whereas in the term neonate, fasting increases hepatic and pulmonary glutathione concentrations. These findings in preterm guinea pigs agree well with earlier observations of only minor decreases in tissue glutathione concentrations following a 72 hr starvation period [14]. Preterm guinea pig neonates, but not full term animals, undergo a transient decrease in tissue GSH concentrations following delivery (S. C. Langley, unpublished observations). Preterm animals are able to reestablish the GSH concentrations present at birth even during conditions of total starvation, and presumably shortage of substrates for GSH synthesis.

The presence of increased hepatic and pulmonary glutathione concentrations following starvation of term neonates is an intriguing observation. A similar response to fasting has been demonstrated in adult hamsters [8, 24]. In hamsters this has been explained, in part, by an increase in GRed activity and a decreased efflux of glutathione into the blood [8]. Such changes are in part true of the term guinea pig pup as GRed activities of both the lung and liver increased substantially, a response which was not observed in either fasted adults or preterm pups. Igarashi *et al.* [8] also demonstrated that fasting did not lead to an increase in hepatic GRed activity of adult guinea pigs, rats or mice, although feeding low-protein diets produced an increase in hepatic GRed activity of rats [11]. Hepatic τGGT activity, which is, in the guinea pig, believed to be involved in hydrolytic degradation of glutathione and the mobilization of its cysteine moiety for use in protein synthesis [3, 19, 25] was decreased in both adult and term guinea pigs. This would suggest initiation of changes in the liver which result in the maintenance of glutathione stores.

Fasting elicited decreases in body weight in term pups and adult animals, but not in preterm animals. Possible effects of dehydration on glutathione status were avoided by providing water in all cages. Term pups were able to take water from drinking bottles. Fasted preterm pups, however, were unable to drink in this way, but clinically significant dehydration did not appear to occur as these animals actually lost less weight than fed preterms. The effectiveness of using surrogate mothers with these animals has been well established (Kelly *et al.*, unpublished observations) and in the present study a normal decrease in body weight following premature delivery was observed. Weight loss normally continues for 48–72 hr prior to the establishment of steady growth, and is attributable largely to loss of water.

In adults, normal glutathione status was not maintained despite reduction of hepatic τGGT activity, the putative enzyme of GSH turnover in guinea pigs. In term pups, a combination of decreased degradation by τGGT and increased recycling of GSH/GSSG appears to have been sufficient to increase tissue glutathione stores, although increased synthesis, which was not determined, cannot be excluded. Preterm pups maintained tissue glutathione concentrations without any alteration of either enzyme activity. Pulmonary GRed activity in these animals was significantly higher than in term pups or adults, while hepatic activity was significantly higher than in adult guinea pigs. τGGT activity was lower in preterm neonates than in term pups or adults. Thus, the high level of recycling activity and low activity of the putative degradation enzyme in the premature animal apparently allow it to resist fasting-induced changes in glutathione status.

Preterm guinea pigs had lower hepatic τGGT activity than term pups. This is consistent with the finding that the rat foetus also has low activity of this enzyme rising shortly before birth [26]. The hepatic τGGT activity of term neonates was lower than that of adult guinea pigs. This differs from previous reports which found τGGT activity in rats to be high at birth and then to decrease steadily over the first week of life [25, 26]. However, the adult guinea pig has been shown to have unusually high τGGT activity [8]. The high τGGT activity of the term rat neonate, has been suggested to facilitate the mobilization of cysteine for protein synthesis immediately following birth, this amino acid being provided by the maternal system *in utero* [26]. As τGGT activity increases towards adulthood in the guinea pig, the function of this enzyme in this species may differ to that in the rat. In the rat, a significant loss of liver glutathione, in the course of normal turnover, is due to glutathione efflux into bile [27]. In the guinea pig, biliary GSH excretion is very low [8], suggesting degradation of the tripeptide is mediated predominantly by τGGT.

Hepatic GPx fell during development between preterm and term neonates, and then increased in adult animals. These observations are consistent with our previous findings [17]. Lung GPx activity did not change significantly with age. Hepatic GPx activity decreased in adults on fasting. In term pups hepatic GPx increased following a 48 hr fast, and in both neonatal groups pulmonary GPx was increased. The significance of these changes in response to fasting is unclear, but similar increases in the GPx activity of intestinal mucosa of fasted rats have been reported [28]. GPx is an enzyme involved in the detoxification of organic hydroperoxides, the products of oxidative stress. Increased GPx activity therefore suggests increased oxidative stress in the fasted animal, perhaps as a consequence of the reduced availability of scavenging antioxidants from the diet, such as vitamin E and ascorbic acid [29]. Alternatively, the increased flux of lipids through the β-oxidation pathway in response to starvation, may yield an excess of oxidized products. The finding of increased malondialdehyde levels (often used as an index of oxidative injury) in the intestinal mucosa of fasted rats [28] would support either hypothesis.

These findings have serious implications with regard to basic assumptions about the interaction of diet and xenobiotics. The current view is that starvation or a diet deficient in sulphur-containing amino acids will impair capacity to detoxify foreign substances, by virtue of decreased hepatic glutathione levels [4, 11, 21]. The present study suggests that this may not be the case in the neonate, where glutathione concentrations are maintained or even increased during fasting, with concomitant increases in GPx and GRed activities. Combined, these changes would enhance both antioxidant defences and detoxification mechanisms.

The premature human infant has been suggested to be at greater risk of hyperoxia-induced lung damage when on minimal schemes of total parenteral nutrition, in which only dextran and salts are provided [30]. Animal studies have provided evidence to support this notion. Adult rats fed low-protein diets [10, 11] and fasted adult mice [3, 4] show reduced tolerance of high oxygen tensions, leading to lung injury and death. These findings were attributed to a diminished glutathione status. However, our own studies with preterm guinea pigs, a more appropriate model of hyperoxic lung injury in the premature infant [15], failed to duplicate these earlier studies [14]. In the context of the current data a reevaluation of the role of glutathione in this clinical situation is appropriate. The effects of milk deprivation in the guinea pig neonate have not been examined previously. It is therefore difficult to draw detailed conclusions from the current data. It is apparent that the function of GSH in the newborn is primarily to provide cysteine for protein synthesis, and it would appear that there is a strong drive to store cysteine as GSH immediately following birth. Our preliminary studies indicate that this effect may be short-lived, lasting up to 4–5 days post-partum (S. C. Langley, unpublished observations) in the case of term pups.

Differences in the response to fasting were noted between term and preterm neonates. These may be explained by the mode of delivery experienced by the pups. Preterm pups were exposed to anaesthesia *in utero*, which may cause the transient decrease in GSH concentrations in the lung and liver, which is not observed in term pups undergoing a normal vaginal delivery. Delivery by caesarian section avoids exposure of the pups to a variety of hormonal changes associated with vaginal delivery, for example a surge in glucocorticoid release. These hormonal changes experienced by the term pups influence a number of metabolic processes which could have effects on the glutathione system. For example, glucocorticoids stimulate gluconeogenesis [31], which would have an effect on the availability of amino acid substrates for GSH synthesis. Cysteine and glycine are conditionally essential in both term and preterm neonates [32]. Relative rates of gluconeogenesis in term and preterm guinea pigs may therefore differ, and underly differences in responses of the GSH system to fasting.

In summary, the response of the hepatic and pulmonary glutathione systems in the guinea pig neonate to starvation differs from that seen in fasted, adult guinea pigs and other rodents. Preterm neonates

maintain normal tissue glutathione concentrations without apparently altering capacity to recycle GSH and GSSG, or adjusting degradative activity. Term neonates increase tissue glutathione concentrations by virtue of increased recycling and decreased degradative activity. Evidently, the neonatal guinea pig has a strong drive towards the maintenance of tissue glutathione concentrations, and this finding may have potential clinical implications.

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