Human Reproduction Vol.18, No.10 pp. 2031–2038, 2003 DOI: 10.1093/humrep/deg414

Galactose toxicity in the rat as a model for premature ovarian failure: an experimental approach readdressed

S.Bandyopadhyay[1], J.Chakrabarti[1], S.Banerjee[2], A.K.Pal[1], S.K.Goswami[2], B.N.Chakravarty[2] and S.N.Kabir[1,3]

[1]Reproductive Biology Research, Indian Institute of Chemical Biology, Jadavpur, Kolkata 700032 and [2]Institute of Reproductive Medicine, Salt Lake, Kolkata 700091, West Bengal, India

[3]To whom correspondence should be addressed. E-mail: snkabir@iicb.res.in

BACKGROUND: The pathophysiological mechanisms underlying premature ovarian failure (POF) are largely unknown. Our objective was to develop a working animal model to explore the pathogenesis of POF. Since galactosaemic women eventually develop POF, we evaluated the potential of experimental galactose toxicity as the proposed model. METHODS: Pregnant rats were fed pellets supplemented with or without 35% galactose from day 3 of conception continuing through weaning of the litters. Female offspring were evaluated for serum levels of galactose and galactose-1-phosphate, growth rate, onset of puberty, reproductive cyclicity, ovarian complement of follicles, hypothalamo-pituitary–ovarian function and follicular response to gonadotrophins. RESULTS: Galactose toxicity delayed the onset of puberty and developed a state of hypergonadotrophic hypoestrogenism. The characteristic low FSH levels at weaning followed by pubertal spurts of gonadotrophins and estradiol (E_2) secretion of the controls was replaced by a sustained high level of FSH and a low level of E_2 under galactose toxicity. The ovary developed with apparently normal or deficient complement of follicles. Ovarian response to exogenous gonadotrophin stimulation was blunted, but the response improved significantly when the stimulation was preceded by pituitary desensitization. CONCLUSION: Experimental galactose toxicity may serve as a model for exploring some of the basic tenets of POF.

Key words: galactosaemia/galactose toxicity/premature ovarian failure/rat model/resistant ovary syndrome

Introduction

Premature ovarian failure (POF), a condition causing amenorrhoea and hypergonadotrophic hypoestrogenism before the age of 40 years, affects 1% of women in the general population. POF was once thought to be an irreversible entity, but it has been demonstrated subsequently that in POF, unlike in menopause, residual ovarian function may remain despite the presence of elevated gonadotrophins. Many authorities therefore divide POF patients into two distinct categories: patients with follicle depletion and patients with follicle dysfunction (Anasti, 1998). The latter condition is characterized by the presence of follicles in the ovary that are unresponsive to gonadotrophins, and is usually referred to as 'resistant ovary syndrome' (ROS) (Wentz, 1996). The general consensus is that ROS is one pathway in the progression to irreversible ovarian failure regardless of the mechanism (Richardson, 1993; Conway, 1997). A substantial number of patients, perhaps belonging to the latter category, experience spontaneous remission (Alper *et al*., 1986). However, except for oocyte donation, no evidence-based effective line of therapy for treatment of the disease is currently available (Fitch *et al*., 1982). The therapeutic modalities currently offered to POF patients are only palliative and supportive, rather than curative. Incomplete knowledge of the precise phenomena underlying the pathogenesis of the disease is the major obstacle in devising effective therapeutic options for POF. The disease demonstrates a wide variety of phenotypes. However, except for genetic disorders or exposure to high dose radiation or chemotherapy, its aetiology remains an enigma and the mechanism of pathogenesis remains largely unknown. Identification of the pathophysiological mechanisms is therefore considered an essential prerequisite for the development of new effective treatment options (Liu *et al*., 2000). However, there are limitations in undertaking in-depth studies in humans, and development of a suitable animal model remains a basic need.

A number of reports document that women with galactosaemia eventually develop POF (Kaufman *et al*., 1988; Waggoner *et al*., 1990; Guerrero *et al*., 2000). The pathophysiological attributes of POF in galactosaemic and other chromosomally competent women are likely to be different, but in both cases the disease possibly progresses through similar clinical pathways (Twigg *et al*., 1996). Therefore, investigation of galactosaemic subjects may provide clues to some natural mechanisms underlying the pathogenesis of POF.

The causal link between galactosaemia and POF is not definitely known. There are suggestions, however, that prema-

ture depletion of ovarian follicular reserve is due to toxic effects of galactose and its metabolites (Cramer *et al*., 1989). Earlier reports have documented that feeding large amounts of galactose, which overpowers the normal capacity of the animal to metabolize this sugar, can produce an animal model of galactose toxicity (Gibson, 1995; Segal, 1995). Rodents exposed to high concentrations of galactose had been the models for the study of gonadal dysfunction in galactosaemia. Adult females and the offspring from dams exposed to galactose were reported to exhibit manifestations of diverse ovarian dysfunction that include delayed onset of puberty, decrease in numbers of ovarian follicles and follicular resistance to gonadotrophins. (Chen *et al*., 1981; Meyer *et al*., 1992). We have demonstrated recently that prenatal exposure to high galactose adversely affects oogonial migration and results in a low initial pool of germ cells in the developing gonads (Bandyopadhyay *et al*., 2003). Parallels can be drawn between these specific gonadal dysfunctions and POF or ROS components of human galactosaemia. With the foregoing considerations in mind, we reasoned that it should be of considerable interest to explore if experimental galactose toxicity can serve as a model for the investigations on POF/ROS. The present investigation demonstrates a composite picture of the ovarian dysfunctions under experimental galactose toxicity in rats, and evaluates its suitability as a model to unveil the pathophysiological mechanisms underlying the development of the disease.

Materials and methods

Chemicals and food

The following materials were commercially available: galactose, pregnant mare's serum gonadotrophin (PMSG), HCG, hyaluronidase, chloramine T, β-nicotinamide adenine dinucleotide (β-NAD), α-D-galactose-1-phosphate (dipotassium salt, type II), *p*-iodonitrotetrazolium violet, diaphorase (*Clostridium kluyveri*) (Sigma Chemical Co., St. Louis, MO), alkaline phosphatase (calf intestine) (New England Biolabs, UK), anti-rabbit γ-globulin (Genei, Bangalore, India), carrier free [^{125}I]Na (Bhaba Atomic Research Centre, Mumbai, India), buserelin acetate (Hoechst Marion Roussel Ltd, UK) and β-galactose dehydrogenase (*Pseudomonas* sp.) (Roche Molecular Biochemicals, Germany). Immunoreagents for LH and FSH radioimmunoassay were gifts from the National Hormone and Pituitary Program, NIDDK, Torrance, CA. The chemiluminescence enzyme immunoassay kit for estradiol (E_2) estimation was purchased from Chiron Diagnostics Corp., MA. Standard food pellets (carbohydrate, 65.5%; protein, 21%; fat, 5.5%; mineral mixture, 7%; and vitamin mixture, 1%), supplemented with or without 35% galactose, were produced in our own animal house.

Animals

The experiments were performed in accordance with the guidelines formulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Culture, India, with approval from the Animal Ethics Committee of the Indian Institute of Chemical Biology. Sprague–Dawley rats maintained under good husbandry conditions of food and water *ad libitum* with a diurnal cycle of 12 h light and 12 h dark starting at 0600 h were raised in our institute animal house. Adult female rats were mated with proven fertile males of the same strain, and the presence of sperm in the vaginal lavage was assigned as day 1 of gestation. They were fed standard food pellets supplemented with (gal-exposed; n = 38) or without (control; n = 24) galactose from day 3 of conception, continuing through weaning of the litters on postnatal day (PND) 21 (PND0 being the day of birth) when the female litters were separated and maintained separately, and employed for the following investigations.

Estimation of galactose and galactose-1-phosphate

Blood levels of galactose and galactose-1-phosphate were measured at weaning (PND21) (control, n = 12; gal-exposed, n = 15) as well as adult age (PND70/71) (control, n = 7; gal-exposed, n = 11). Blood was collected by direct cardiac puncture; sera were procured after centrifugation and assayed for simultaneous determination of galactose and galactose-1-phosphate by a colorimetric microassay method. The assay protocol was a modification of that described by Diepenbrock *et al*. (1992), wherein measured volumes of sera and standards were used instead of dried blood spots and standards. Measurement was done in the form of galactose alone or with that released from galactose-1-phosphate following co-incubation with alkaline phosphatase and galactose dehydrogenase. The concentrations were expressed as mmol/l.

Assessment of growth rate

If not otherwise mentioned, body weights (BWs) of the litters were recorded every 5th day since birth, and continued throughout the course of the experiments.

Assessment of onset of puberty

The onset of puberty was marked by the time of vaginal opening (VO). From PND35 onward, the rats (control, n = 30; gal-exposed, n = 57) were checked daily for VO. On the day of VO, age and BW of the respective rats were recorded, and during the subsequent course of investigations estrous cyclicity was evaluated by vaginal cytology.

Assessment of ovarian follicular reserve

Ovaries of the 55- to 60-day-old animals were dissected out immediately after sacrifice, trimmed of fat, weighed and immerse-fixed in Bouin's fixative for 24 h followed by paraffin embedding and subsequent processing for routine histopathological evaluation under light microscopy.

Assessment of pituitary gonadal functions

Either at weaning on PND21 or on PND42/43, rats were euthenized. Blood was collected by direct cardiac puncture, and serum was separated and stored at –70°C for subsequent determination of LH, FSH and E_2.

Serum levels of LH and FSH were measured by a double-antibody radioimmunoassay method. Pure rat LH (NIDDK-rLH-I-9) and FSH (NIDDK-rFSH-I-9) were iodinated with carrier-free ^{125}I using chloramine T according to the method of Greenwood *et al*. (1963). The antisera to LH and FSH were NIDDK-anti-rLH-S-11 and NIDDK-anti-rFSH-S-11, respectively. The sensitivities of the assays were 0.15 ng/ml for LH and 0.5 ng/ml for FSH. All samples were assayed in a single batch at two concentrations, and each in duplicate. The intra-assay coefficients of variations were <8%. The LH and FSH values were expressed in terms of the reference standards, NIDDK-rat-LH-RP-3 and NIDDK-rat-FSH-RP-2.

Serum levels of E_2 were measured by fully automated chemiluminescence assay system, ACS 180 (Chiron Diagnostics Corp., East Walpole, MA). The sensitivity of the assay was 10 pg/ml and the intra-assay coefficient of variation was 5.6%.

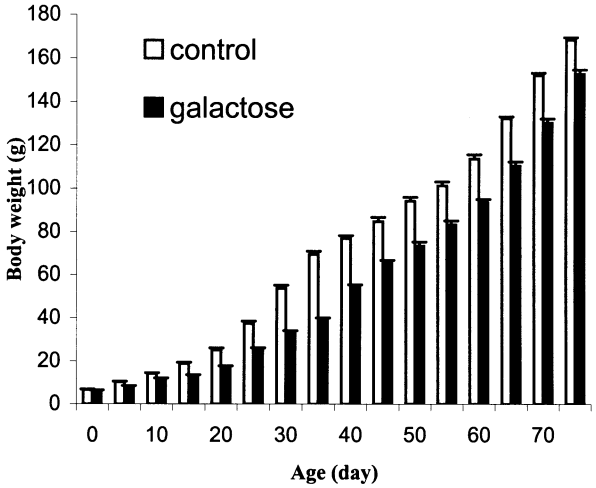

Figure 1. Postnatal growth rates in control and galactose-exposed litters. Both groups show a steady increase in their body weight throughout the course of study, but the galactose-exposed group exhibits a remarkably poor growth rate with no characteristic peri-pubertal growth spurt. The number of rats used in each point of evaluation was between 15 and 22.

Assessment of follicular response to gonadotrophins

Between PND25 and PND30, control ($n = 25$) and gal-exposed ($n = 35$) rats were subjected to induction of the superovulatory response by exogenous gonadotrophins. They had an s.c. injection of 30 IU of PMSG at 0 h. A subset of animals from each group (control, $n = 10$; gal-exposed, $n = 14$) were sacrificed at 50 h when blood was collected, and serum was procured and stored frozen at –70°C until assessed for E_2 levels. The remaining animals had an s.c. injection of 50 IU of HCG at 52 h followed by autopsy at 72 h when the presence of oocytes in the oviduct was assessed and counted. In another set of control ($n = 15$) and gal-exposed rats ($n = 23$), superovulation was effected after induction of pituitary desensitization. The rats had an s.c. injection of 100 µg of GnRH agonist per day in two equal doses for 10 days, and were then subjected to the induction of superovulation by PMSG–HCG. The ovulatory response was assessed with respect to both numbers of rats exhibiting the presence of ovulated oocytes and the numbers of oocytes ovulated per rat stimulated.

Statistics

The data were expressed as mean ± SEM, and n refers to the number of animals or determinations. Differences between the groups were analysed by one-way ANOVA, Student's t-test and χ^2 tests, as applicable, using the Epistat package (Tracy L.Gustafson, 2011 Cap Rock Circle, Richardson, TX 75080).

Results

Blood levels of galactose and galactose-1-phosphate

The study population had significantly elevated levels of galactose (mmol/l) and galactose-1-phosphate (mmol/l) at weaning (galactose, 0.35 ± 0.01; galactose-1-phosphate, 0.07 ± 0.01) as well as at 70/71 days of age (galactose, 0.38 ± 0.02; galactose-1-phosphate, 0.05 ± 0.01), compared with almost non-detectable levels of both analytes in the respective control groups.

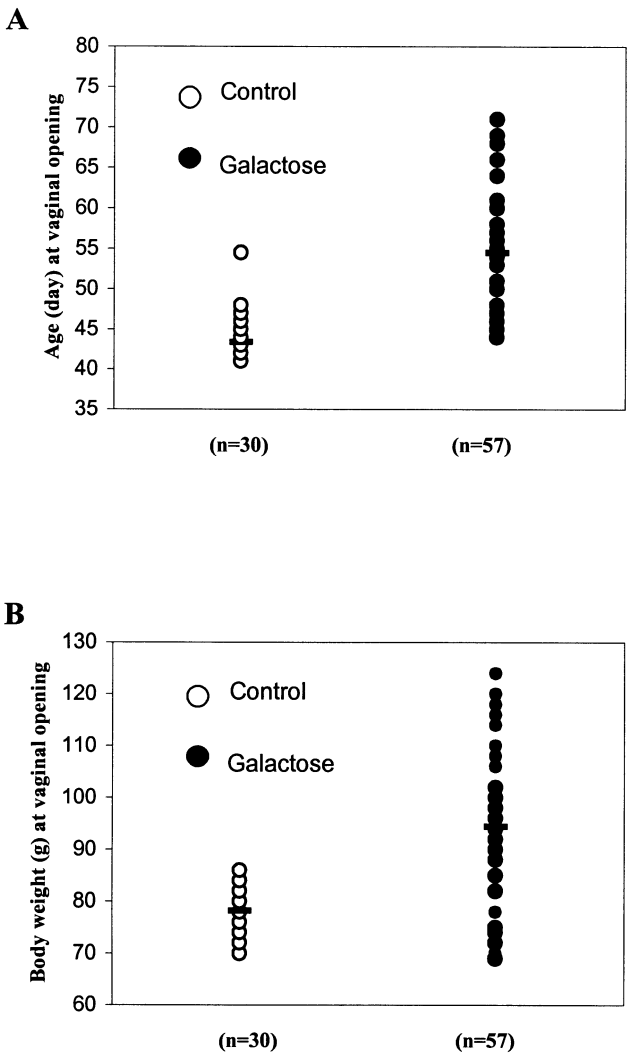

Figure 2. Timing of vaginal opening in control and galactose-exposed litters in relation to their age distribution (**A**) and body weight (**B**). CI is the 95% confidence interval and (n) is the number of rats used. For age, the galactose group (CI 38.3–70.7) were significantly different ($P = 0.000001$) from control (CI 38.5–48.3). For body weight, the galactose group (CI 60.8–127.9) were significantly different ($P = 0.000002$) from control (CI 66.8–89.6)

Growth rate

BWs of the litters at birth and subsequent growth rates are presented in Figure 1. At birth, the BWs of the pups were not statistically different, although the gal-exposed pups had relatively lower weight. Though both groups exhibited a steady rise in their BW, the rate of growth was significantly lower in the study population throughout the period of study. The difference in the growth rates reached the maximum level of significance during PND30–40 when the control group exhibited a pre-pubertal growth spurt.

Vaginal opening

The timing of VO has been expressed in relation to age (Figure 2A) as well as BW (Figure 2B). The mean age (days) (control 43.4 ± 0.45 versus gal-exposed 54.5 ± 1.10) and BW (g) (control 78.2 ± 1.06 versus gal-exposed 94.4 ± 2.26) on

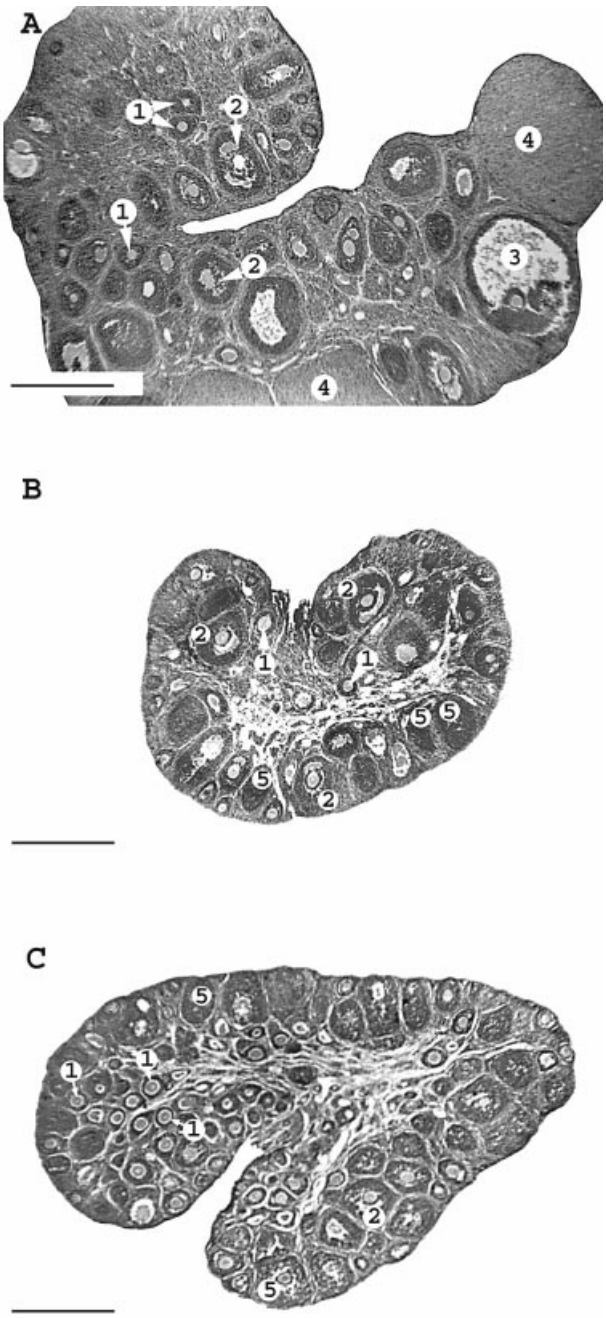

Figure 3. Histological sections through ovaries of 55- to 60-day old control (**A**) and galactose-exposed rats (**B** and **C**). The control ovary exhibits the presence of follicles at different stages of maturity and a functional corpus luteum (CL). The galactose-exposed ovaries exhibit an infantile appearance with a deficient state of follicular reserve (B), or the presence of apparently normal complement of unstimulated follicles arrested mostly at the pre-antral stage and a preponderance of atretic follicles (C). The absence of graafian follicles and CL was the most salient feature. 1, pre-antral or secondary follicles; 2, early antral follicles or vesicular follicles; 3, graafian follicle; 4, corpus luteum; 5, atretic follicles. Bars = 50 μm.

the day of VO was significantly ($P \leqslant 0.000002$) higher in the study group than in the control group. With a 95% confidence interval (CI), the age of 67% of the gal-exposed litters on the day of VO was beyond the upper confidence limit of the control population. In 63% of the gal-exposed rats, VO was delayed although they exceeded the upper BW limit of the control population at the time of VO.

Compared with 4 or 5 day regular estrous cycles in the control group, the gal-exposed group exhibited marked irregularity in the cycles as characterized by increased length, ranging from 8 to 11 days mostly due to prolongation of the di-estrous phase, with the sporadic appearance of pro-estrus followed by estrus.

Ovarian histology

Ovarian sections of control rats (Figure 3A) showed the presence of follicles at different stages of maturation and the corpus luteum (CL). In contrast, the study population exhibited diverse architecture of the ovaries (Figure 3B and C). Reduced ovarian mass and absence of graafian follicle and CL were generalized findings. However, many ovaries were deficient in small follicles (Figure 3B), while the others had an infantile appearance with a normal complement of follicles but with growth and differentiation arrested at the early pre-antral stage (Figure 3C). The presence of a large number of pyknotic granulosa cells, arranged in a single symmetric ring adjacent to the basement membrane and offering the structure of a string of beads, marked the preponderance of atretic cells (Figure 3B).

Serum levels of gonadotrophins and E_2

Hormone levels are summarized in Table I. The control rats had characteristic low FSH and high E_2 levels at weaning, which was followed by a spurt of FSH and E_2 secretion at the stipulated peri-pubertal phase. The study group, on the other hand, had significantly lower levels of E_2 and elevated gonadotrophins on both occasions.

Ovarian response to induction of superovulation

Table II depicts the results of the ovarian response to gonadotrophins. Compared with the control group, the ovarian response to PMSG–HCG with respect to the number of rats ovulated or the rate of oocyte retrieval, and the peak E_2 value on the day of HCG administration were significantly poorer in the study group. When gonadotrophin stimulation was preceded by pituitary desensitization, the ovarian response in the study group improved over its corresponding control group in all respects. However, while the number of rats responding to stimulation (82.6%) almost reached the GnRH agonist control level (86.6%), the oocyte retrieval rate still remained significantly lower ($P = 0.013$).

Discussion

To devise effective therapeutic options for POF, further appreciation of the pathophysiological mechanisms of the disease is of prime importance. We therefore decided to develop a suitable animal model that may serve to explore the pathogenesis of the disease.

Women with galactosaemia eventually develop POF (Kaufman *et al.*, 1988; Waggoner *et al.*, 1990; Guerrero *et al.*, 2000). It has been proposed that the pathogenesis of POF in galactosaemic and other women possibly progresses through similar clinical pathways (Twigg *et al.*, 1996). Thus, investi-

Table I. Serum levels of LH, FSH and estradiol at weaning (PND21) and peri-pubertal phase (PND42/43)

Group	LH (ng/ml)		FSH (ng/ml)		E_2 (pg/ml)	
	PND21	PND42/43	PND21	PND42/43	PND21	PND42/43
Control	0.70 ± 0.09[a] (8)	3.42 ± 0.18[b] (12)	4.10 ± 0.30[d] (8)	6.67 ± 0.85[e] (12)	25.63 ± 1.77[g] (8)	35.34 ± 1.76[i] (12)
Gal-exposed	0.91 ± 0.07[a] (13)	6.40 ± 0.50[c] (21)	7.25 ± 0.53[e] (13)	19.79 ± 1.80[f] (21)	12.48 ± 1.11[h] (13)	13.20 ± 0.70[h] (21)

Numbers within parentheses denote numbers of determinations in the corresponding group.
Values are mean ± SEM.
LH values with different superscripts differ significantly at $P \leqslant 0.0001$ levels.
FSH values differ significantly at the levels of $P \leqslant 0.02$, d versus e; $P < 0.0001$, e versus f.
E_2 values with different superscripts differ significantly at $P \leqslant 0.001$ levels.

Table II. Ovarian response to PMSG–HCG stimulation with and without pituitary desensitization

Group	Without pituitary desensitization		With pituitary desensitization	
	Control	Gal-exposed	Control	Gal-exposed
Rats ovulated (%)	100[a] (15)	61.9[b] (21)	86.6[a] (15)	82.6[a] (23)
Oocyte retrieval rate	22.1 ± 2.6[c] (15)	8.6 ± 1.9[d] (21)	22.7 ± 2.7[c] (15)	15.0 ± 1.6[e] (23)
E_2 (pg/ml)	395 ± 69[f] (10)	165 ± 40[g] (14)	NE	NE

Numbers within parentheses denote numbers of determinations in the corresponding group.
Values are mean ± SEM.
NE = not estimated.
Values in a row with different superscripts differ significantly: a versus b, $P < 0.05$; c versus d, $P = 0.0001$; c versus e, $P = 0.013$; f versus g, $P = 0.005$.

gations on galactosaemic subjects are likely to unravel some natural mechanisms that may underlie the development of POF (Twigg *et al*., 1996).

There is good evidence that galactose readily crosses the placenta (Segal, 1995). Administration of galactose to rats during pregnancy produces a sequela of abnormalities in the litters which are characteristic of human galactosaemia (Segal and Bernstein, 1963; Spatz and Segal, 1983). Thus the rodents provide an excellent model system for galactose toxicity, which is easy to acquire, maintain and modulate. We thought it reasonable to evaluate the potentiality of this experimental condition as the proposed model for the present investigation.

Increased levels of galactose and its metabolites are thought to be responsible for most of the toxic manifestations of galactosaemia. To assess the magnitude of galactose toxicity under the prevailing experimental condition with our treatment regimen, we measured the levels of galactose and galactose-1-phosphate in the circulation. As expected, both were found to be significantly elevated at weaning as well as adult age.

Little information is available on pre-pubertal growth in chromosomally competent women who subsequently developed POF. However, retrospective analyses of large series of galactosaemic POF women showed that many affected women had severely delayed growth during childhood and early adolescence, although normal growth and development was also very common (Waggoner *et al*., 1990). In a rat model, Chen *et al*. (1984) demonstrated that galactose toxicity had an adverse impact on intra-uterine embryonic growth. The present investigation, however, documents only a smaller and insignificant reduction of mean litter weight at birth, although subsequent growth rates were significantly retarded. This differential growth rate among different galactosaemic women or between the experimental models may be attributed to the difference in magnitude of galactose toxicity. We had observed earlier that feeding rats with a 50% galactose diet, the dosage at which Chen *et al*. (1981) observed a significantly reduced birth weight of the pups, was associated with a significant reduction in the BW of the dams, and an increased incidence of in-utero fetal resorption and stillbirths. We therefore gradually minimized the galactose content of the diets and considered 35% galactose as the optimum level since it exerted no adverse effect on the mothers' BW and mean litter size was comparable with that of the controls.

With regard to the onset of puberty, there are a number of pertinent observations. Delayed puberty is a frequent finding in women with galactosaemia (Fraser *et al*., 1986). Late onset of puberty under experimental galactose toxicity has been reported previously (Chen *et al*., 1981). The present results also document that the timing of VO, a reproductive tract response to ovarian steroids and one of the key indices for puberty in rodents, was significantly delayed under galactose toxicity. The onset of puberty involves a chain of events including increased production of sex steroids by the gonads in response to increased secretion of gonadotrophins from the anterior pituitary. This is driven by a spurt in the secretion of GnRH following activation of the hypothalamus. Exactly when

the puberty-specific increase in the cascade of hormones begins largely depends on body size, rather than chronological age (Foster and Nagatani, 1999). Since the gal-exposed litters had a retarded growth rate, it may be argued that delayed onset of puberty was not a direct effect of galactose toxicity, but rather attributed to delayed growth rate. To resolve the issue, we evaluated VO separately in relation to age and BW. We observed that 63% rats of the study group did not exhibit VO despite their BWs being within the 95% CI of the controls at the time of VO. This observation suggests that galactose toxicity *per se* is responsible for delayed puberty. Whatever the mechanism, delayed puberty generally results from an ultimate lag in activation of one or more of the factors determining the maturation of the hypothalamo-pituitary–ovarian (HPO) axis (Rosenfield, 1996). Observation of elevated gonadotrophins with low circulating levels of E_2 in the study group, however, discounts the possibility of inactivation at the hypothalamo-pituitary level and points to the ovary as the possible primary target of galactose toxicity.

In immature female rats, FSH secretion remains high until PND18 and decreases thereafter (Thyssen and Libertun, 1996). A subsequent rise in FSH level occurs at the time of VO. This sequential drop and rise of FSH secretion is likely to be of phenomenal importance in the process of onset of puberty. Extended central nervous system (CNS) restraint on GnRH release is considered an attractive hypothesis for delayed puberty (Rosenfield, 1996), but prolongation of high FSH by inhibition of polyamine synthesis also delays the onset of puberty independently of body mass (Thyssen and Libertun, 1996). A drop in FSH during PND18 has been suggested to be a steroid-independent event of cerebral origin, while the rise of gonadotrophins that immediately precedes VO is brought about by a pre-pubertal gradual rise in estrogen secretion that acts at the CNS–pituitary level to trigger a pre-ovulatory pro-estrous-like surge of gonadotrophins (Ojeda *et al*., 1976). We demonstrated that the gal-exposed litters had elevated FSH levels both at weaning and at the stipulated peri-pubertal phase; and E_2 levels remained significantly low throughout. It is therefore tempting to speculate that the absence of stimuli in the form of E_2 perhaps blunts the puberty-specific sensitization of the CNS–pituitary unit.

Food restriction during late gestation and lactation in rats has been reported to have negative impacts on the HPO axis, and to prohibit follicular maturation and delay the onset of puberty (Leonhardt *et al*., 2003). That the rats maintained on our dietary regimen were not underfed or malnourished has been substantiated in our earlier report (Bandyopadhyay *et al*., 2003). Moreover, we also maintained the protein content of the galactose diet at 13.65% so that the basic protein requirement (12%) for pregnant rats is met. Therefore, the possibility of any implications of reduced food intake and consequent malnutrition on delayed puberty and related endocrinopathies under galactose toxicity can be ruled out.

Galactosaemia encompasses a broad spectrum of ovarian pathology. While some patients clearly demonstrate ovarian failure related to follicle depletion, others have primordial follicles appearing normal in ovarian biopsy but lacking growth and development (Fraser *et al*., 1986). In order to typify the ovarian effects of galactose toxicity, we investigated the ovarian follicular reserve and also evaluated its response to gonadotrophins. Chen *et al*. (1981) reported that prenatal galactose toxicity led to a significant reduction in the number of small follicles. Our histopathological examination, however, did not reveal such a generalized effect. While many rats suffered from deficient follicular reserve, others exhibited apparently normal follicular complement, though they were unresponsive to exogenous gonadotrophins. Thus the basic tenets of the diverse phenotypes of POF are also conserved in experimental galactose toxicity.

The size of a follicular complement at any point of reproductive life represents the balance between the numbers of follicles endowed in the initial ovarian pool and those expended since then. A deficient follicular reserve may therefore ensue from an under-endowment of follicles in the ovarian pool and/or an accelerated rate of atresia—the major form of follicular expenditure. Within the reproductive system, the ovary is considered to be one of the major targets of galactose metabolites that have detrimental effects on the germ cells, granulosa cells, stromal cells and thecal cells (Kaufman *et al*., 1987, 1989; Gibson, 1995). Fraser *et al*. (1986) demonstrated that ovarian accumulation of galactose-1-phosphate led to degradation of uridine nucleotides and induced follicular atresia. In the present study, we observed a general preponderance of atretic secondary follicles in the gal-exposed group. On the other hand, we have demonstrated recently that galactose toxicity adversely affected germ cell migration leading to ovary development with a low initial pool of germ cells (Bandyopadhyay *et al*., 2003). We therefore suggest that an increased rate of atresia as well as a deficient initial ovarian pool of germ cells are implicated in the process of premature depletion of follicles under galactose toxicity.

The state of hypergonadotrophic hypoestrogenism in the presence of an apparently normal complement of follicles under galactose toxicity attests to the state of follicular refractoriness to gonadotrophins. There are, however, suggestions that in galactosaemic women, some aberrant forms of gonadotrophins are produced that are biologically inactive (Prestoz *et al*, 1997). In order to replace such putative defective gonadotrophins, we induced superovulation in rats by exogenous gonadotrophins. Ovarian response, expressed in terms of the number of rats exhibiting ovulation or the number of oocytes ovulated per rat, was significantly poorer in the study group. It was not surprising that ovaries with deficient follicles responded poorly to gonadotrophins. However, it was significant that post-autopsy histological analysis of the gonadotrophin-exposed ovaries showed them to possess an apparently normal complement of unstimulated follicles (data not shown). If reduced biopotency of the endogenous gonadotrophins were the sole factor responsible for follicular refractoriness, these follicles should have responded to the exogenous gonadotrophins. In order to look for additional factor(s), we carried out the induction of superovulation after pituitary desensitization with chronic administration of GnRH agonist. The objective was to assess the ovarian response to exogenous gonadotrophins without any intervening effects of endogenous gonadotrophins. A significant increase in ovarian response in the

GnRH-downregulated group raises the possibility that endogenous gonadotrophins had also exerted anti-gonadotrophic activity, presumably at the receptor level. It may, however, be relevant in this context that in galactosaemic patients, some neutral isoforms of FSH are produced that are partially deficient in galactose and sialic acid in the carbohydrate side chains (Prestoz *et al*., 1997). These altered forms of FSH are characterized by their higher binding affinity for FSH receptors but reduced ability to activate the second messenger system. By virtue of this property, these isoforms of FSH may act as anti-hormones and produce antagonistic effects at the receptor level (Sairam, 1989).

Earlier workers have proposed some genetically engineered mouse models for POF. Pellas *et al*. (1991) described an insertional mutation in mouse that caused a deficiency of primordial germ cells reaching the genital ridge during embryonic development. Dong *et al*. (1996) effected targeted deletion using stem cell technology to produce growth differentiation factor-9 (GDF-9)-deficient mice that had unhindered formation of primordial and primary follicles but suffered from follicular development beyond the primary one-layer stage. Both of these experimental conditions could potentially be valuable animal model for human POF. However, if the diverse phenotypes of POF are taken into consideration, each model has its own limitations. A germ cell-deficient mouse may be reminiscent of only the phenotype of the follicle depletion type of POF, while a GDF-9-deficient mouse has the potential to be a model for the follicle dysfunction type of POF. However, galactose toxicity is unique in that it encompasses both spectra of the disease.

Finally, diverse aetiology and multifaceted phenotypes of POF limit the potential of a single model in encompassing the full spectrum of the pathogenesis of the disease. Importantly, however, in view of the foregoing, we are left with the conclusion that experimental galactose toxicity has the credentials to serve as a model at least to explore some of the basic tenets of POF/ROS irrespective of aetiology.

Acknowledgements

This communication was presented in part at the International Symposium on Molecular and Clinical Aspects of the Regulation of Ovarian Function: Impact on the Reproductive Health of Women, New Delhi, India, 1999; and 34th Annual Meeting of Society for the Study of Reproduction, Ottawa, Canada, 2001. We thank NIDDK, California, USA for generously providing the rLH and rFSH immunoreagents for radioimmunoassay. H.N.Ray, Indian Institute of Chemical Biology, is gratefully acknowledged for his assistance in photography. This study was supported by grants from the Department of Science and Technology (SP/SO/B31/97), New Delhi, India.

References

Alper, M.M., Jolly, E.E. and Garner, P.R. (1986) Pregnancies after premature ovarian failure. *Obstet. Gynecol.*, **67** (Suppl. 3), S59–S62.

Anasti, J.N. (1998) Premature ovarian failure: an update. *Fertil. Steril.*, **70**, 1–15.

Bandyopadhyay, S., Chakrabarti, J., Banerjee, S., Pal, A. K., Bhattacharyya, D., Goswami, S.K., Chakravarty, B.N. and Kabir, S.N. (2003) Prenatal exposure to high galactose adversely affects initial gonadal pool of germ cells in rats. *Hum. Reprod.*, **18**, 276–282.

Chen, Y.T., Mattison, D.R., Feigenbaum, L., Fukui, H. and Schulman, J.D. (1981) Reduction in oocyte number following prenatal exposure to a diet high in galactose. *Science*, **214**, 1145–1147.

Chen, Y.T., Mattison, D.R., Bercu, B.B. and Schulman, J.D. (1984) Resistance of the male gonad to a high galactose diet. *Pediatr. Res.*, **18**, 345–348.

Conway, G.S. (1997) Premature ovarian failure. *Curr. Opin. Obstet. Gynecol.*, **9**, 202–206.

Cramer, D.W., Harlow, B.L., Barbieri, R.L. and Ng, W.G. (1989) Galactose-1-phosphate uridyl transferase activity associated with age at menopause and reproductive history. *Fertil. Steril.*, **51**, 609–615.

Diepenbrock, F., Heckler, R., Schickling, H., Engelhard, T., Bock, D. and Sander, J. (1992) Colorimetric determination of galactose and galactose-1-phosphate from dried blood. *Clin. Biochem.*, **25**, 37–39.

Dong, J., Albertini, D.F., Nishimori, K., Kumar, T.R., Lu, N. and Matzuk, M.M. (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature*, **383**, 531–535.

Fitch, N., de Saint Victor, J., Richer, C.L., Pinsky, L. and Sitahal, S. (1982) Premature menopause due to a small deletion in the long arm of the X chromosome: a report of three cases and a review. *Am. J. Obstet. Gynecol.*, **142**, 968–972.

Foster, D.L. and Nagatani, S. (1999) Physiological perspectives on leptin as a regulator of reproduction: role in timing puberty. *Biol. Reprod.*, **60**, 205–215.

Fraser, I.S., Russell, P., Greco, S. and Robertson, D.M. (1986) Resistant ovary syndrome and premature ovarian failure in young women with galactosaemia. *Clin. Reprod. Fertil.*, **4**, 133–138.

Gibson, J.B. (1995) Gonadal function in galactosemics and in galactose-intoxicated animals. *Eur. J. Pediatr.*, **154** (Suppl. 2), S14–S20.

Greenwood, F.C., Hunter, W.M. and Glover, J.S. (1963) The preparation of ^{131}I-labelled human growth hormone of high specific activity. *Biochem. J.*, **89**, 114–123.

Guerrero, N.V., Singh, R.H., Manatunga, A., Berry, G.T., Steiner, R.D. and Elsas, L.J. (2000) Risk factors for premature ovarian failure in females with galactosemia. *J. Pediatr.*, **137**, 833–841.

Kaufman, F.R., Donnell, G.N. and Lobo, R.A. (1987) Ovarian androgen secretion in patients with galactosemia and premature ovarian failure. *Fertil. Steril.*, **47**, 1033–1034.

Kaufman, F.R., Xu, Y.K., Ng, W.G. and Donnell, G.N. (1988) Correlation of ovarian function with galactose-1-phosphate uridyl transferase levels in galactosemia. *J. Pediatr.*, **112**, 754–756.

Kaufman, F.R., Xu, Y.K., Ng, W.G., Silva, P.D., Lobo, R.A. and Donnell, G.N. (1989) Gonadal function and ovarian galactose metabolism in classic galactosemia. *Acta Endocrinol.* (*Copenh*), **120**, 129–133.

Leonhardt, M., Lesage, J., Croix, D., Dutriez-Casteloot, I., Beauvillain, J.C. and Dupouy, J. P. (2003) Effects of perinatal maternal food restriction on pituitary–gonadal axis and plasma leptin level in rat pup at birth and weaning and on timing of puberty. *Biol. Reprod.*, **68**, 390–400.

Liu, G., Hale, G.E. and Hughes, C.L. (2000) Galactose metabolism and ovarian toxicity. *Reprod. Toxicol.*, **14**, 377–384.

Meyer, W.R., Doyle, M.B., Grifo, J.A., Lipetz, K.J., Oates, P.J., DeCherney, A.H. and Diamond, M.P. (1992) Aldose reductase inhibition prevents galactose-induced ovarian dysfunction in the Sprague–Dawley rat. *Am. J. Obstet. Gynecol.*, **167**, 1837–1843.

Ojeda, S.R., Wheaton, J.E., Jameson, H.E. and McCann, S.M. (1976) The onset of puberty in the female rat: changes in plasma prolactin, gonadotropins, luteinizing hormone-releasing hormone (LHRH), and hypothalamic LHRH content. *Endocrinology*, **98**, 630–638.

Pellas, T.C., Ramachandran, B., Duncan, M., Pan, S.S., Marone, M. and Chada, K. (1991) Germ-cell deficient (gcd), an insertional mutation manifested as infertility in transgenic mice. *Proc. Natl Acad. Sci. USA*, **88**, 8787–8791.

Prestoz, L.L.C., Couto, A.S., Shin, Y.S. and Petry, K.G. (1997) Altered follicle stimulating hormone isoforms in female galactosaemia patients. *Eur. J. Pediatr.*, **156**, 116–120.

Richardson, S.J. (1993) The biological basis of menopause. In Burger, H.G. (ed.), *Clinical Endocrinology and Metabolism: The Menopause*. W.B. Saunders Company, London, Vol. **17**, pp. 1–16.

Rosenfield, R.L. (1996) Delayed puberty. In Adashi, E.Y., Rock, J.A. and Rosenwaks, Z. (eds), *Reproductive Endocrinology, Surgery, and Technology*. Lippincott-Raven Publishers, Philadelphia, PA, Vol. **2**, pp. 1007–1015.

Sairam, M. R. (1989) Role of carbohydrates in glycoprotein hormone signal transduction. *FASEB J.*, **3**, 1915–1926.

Segal, S. (1995) *In utero* galactose intoxication in animals. *Eur. J. Pediatr.*, **154** (Suppl. 2), S82–S86.

Segal, S. and Bernstein, H. (1963) Observations on cataract formation in the newborn offspring of rats fed a high-galactose diet. *J. Pediatr.*, **62**, 363–370.

Spatz, M. and Segal, S. (1983) Transplacental galactose toxicity in rats. *J. Pediatr.*, **67**, 438–446.

Thyssen, S.M. and Libertun, C. (1996) Alpha-difluoromethylornithine modifies FSH secretion and puberty onset in the female rat. *Proc. Soc. Exp. Biol. Med.*, **211**, 76–80.

Twigg, S., Wallman, L. and McElduff, A. (1996) The resistant ovary syndrome in a patient with galactosemia: a clue to the natural history of ovarian failure. *J. Clin. Endocrinol. Metab.*, **81**, 1329–1331.

Waggoner, D.D., Buist, N.R. and Donnell, G.N. (1990) Long-term prognosis in galactosaemia: results of a survey of 350 cases. *J. Inherit. Metab. Dis.*, **13**, 802–818.

Wentz, A.C. (1996) Resistant ovary syndrome. In Adashi, E.Y., Rock, J.A. and Rosenwaks, Z. (eds), *Reproductive Endocrinology, Surgery, and Technology*. Lippincott-Raven Publishers, Philadelphia, PA, Vol. **2**, pp. 1385–1392.

Submitted on October 7, 2003; resubmitted on May 21, 2003; accepted on June 30, 2003