Infant feeding with soy formula milk: effects on puberty progression, reproductive function and testicular cell numbers in marmoset monkeys in adulthood

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BACKGROUND: This marmoset study addresses concerns about feeding human male infants with soy formula milk (SFM). METHODS: From age 4 to 5 days, seven male co-twin sets were fed standard formula milk (SMA) or SFM for 5–6 weeks; blood samples were subsequently collected at 10-week intervals. Testes from co-twins killed at 120–138 weeks were fixed for cell counts. RESULTS: SFM- and SMA-fed twins showed normal weight gain; puberty started and progressed normally, based on blood testosterone measurements. Body weight, organ weights (prostate, seminal vesicles, pituitary, thymus and spleen) and penis length were comparable in co-twins. All SMA- and 6/7 SFM-fed males were fertile. Unexpectedly, testis weight (P = 0.041), Sertoli (P = 0.025) and Leydig cell (P = 0.026) numbers per testis were consistently increased in SFM-fed co-twins; the increase in Leydig cell numbers was most marked in males with consistently low-normal testosterone levels. Seminiferous epithelium volume per tubule showed a less consistent, non-significant increase in SFM-fed males; raised germ cell numbers per testis, probably due to increased Sertoli cells, conceivably resulted in larger testes. Average lumen size, although greater in SFM-fed group, was inconsistent between co-twins and the difference was not significant. CONCLUSIONS: Infant feeding with SFM has no gross adverse reproductive effects in male marmosets, though it alters testis size and cell composition, and there is consistent, if indirect, evidence for possible ‘compensated Leydig cell failure’. Similar and perhaps larger changes likely occur in adult men who were fed SFM as infants.

Key words: Leydig and Sertoli cells/marmoset/puberty and fertility/soy formula milk/testosterone

Introduction

In Western countries, many infants are fed with formula milk instead of being breastfed. Although feeding with formula milk was restricted initially to cow’s milk-based formulae, within the last half century it has become common in some parts of the world for infants to be fed with soy formula milk (SFM) (Polack et al., 1999). In most European countries, feeding with SFM is restricted to infants in whom intolerance to other formula milks has occurred, and as a result the prevalence of SFM feeding in these countries is <2% (UK Working Group on Phytoestrogens and Health, 2003). In contrast, in the USA, the prevalence of feeding with SFM at any stage after birth can be as high as 36% (Merritt and Jenks, 2004), and many of these infants are fed with SFM from soon after birth and throughout infancy (Polack et al., 1999). There has been an intense debate as to whether or not feeding with SFM poses a potential health risk to infants (Badger et al., 2002; Mendez et al., 2002; Chen and Rogan, 2004; Merritt and Jenks, 2004). As a consequence of this debate, there have been health recommendations in some countries that SFM should only be used when all other alternatives have been ruled out (Australian College of Paediatrics, 1998; Ministry of Health, Wellington, New Zealand, 1998; COMA UK, 2000). In contrast, in the USA, the American Academy of Paediatrics did not recommend any restriction on the use of SFM (American Academy of Paediatrics Committee on Nutrition, 1998).

The lack of agreement between health recommendations in USA and elsewhere is largely a reflection of uncertainty about the available data that have assessed whether or not SFM, or components of SFM such as the phytoestrogens genistein and daidzein, pose any health risk (Badger et al., 2002; Tuohy, 2003). Much of the evidence pointing to the potential adverse effects of SFM or phytoestrogens derives from studies in laboratory animals, mainly rodents, with some of these studies indicating that there may be impaired reproductive development and function (Irvine et al., 1998a; Badger et al., 2002; Chen and Rogan, 2004) or impaired development of the immune system after phytoestrogen exposure perinatally (Yellayi et al., 2002).
2002, 2003). Prompted by the latter findings, recent studies in human infants reared on SFM have revealed no evidence of impaired development of the immune system or of the ability to generate an appropriate antibody response to vaccination (Cordle et al., 2002; Ostrom et al., 2002). Similarly, one retrospective study of infants fed with SFM (Strom et al., 2001) concluded that there was no evidence for any detectable adverse effects on reproductive development. However, this study was conducted by telephone interview and did not involve any direct measurements of hormone levels or of reproductive function in the individuals concerned and has been criticized for a number of reasons (Goldman et al., 2001).

Apart from potential species differences in response to SFM or its components, there are two additional problems related to the safety evaluation of exposure to soy in infancy. The first, which relates only to the human studies, is that detailed assessment of reproductive development, in particular the development of cell types within the testis and their normal function, either requires invasive techniques or must await attainment of adulthood and the direct testing of fertility potential. Invasive approaches are precluded for ethical reasons, and so far no direct assessments of fertility potential or of other reproductive health parameters (e.g. prostatic disease) have been undertaken in adult men or women who have been reared as infants on SFM. The second issue, which relates to laboratory animal studies, is that some of these have either administered components of SFM such as genistein and/or have used a route of administration that is not directly relevant to human formula-fed infants, such as subcutaneous or intraperitoneal injection, or maternal dietary administration of the test material (Mendez et al., 2002; Chen and Rogan, 2004). On the other hand, some of these studies have highlighted that abnormalities resulting from phytoestrogen (genistein) exposure in rodents may not emerge until later in adulthood (Newbold et al., 2001; Jefferson et al., 2003), a period for which there is no relevant information from human studies.

There is therefore uncertainty about whether the studies performed in laboratory animals are of direct relevance to human concerns about SFM-fed infants. Another difference, which contributes to this problem, is that the period in which human male infants are fed with SFM encompasses the neonatal period of testicular activity, during which pituitary and testicular (testosterone and inhibin B) reproductive hormones reach adult levels (Mann and Fraser, 1996; Andersson et al., 1998; Grumbach, 2005). In contrast, in laboratory rodents, this period of neonatal testicular activity is confined to around the day of birth (Corbier et al., 1992). Taken together, these differences cloud an accurate assessment of whether or not feeding human infants with SFM could have potentially adverse or even beneficial effects.

To sidestep the problems outlined above, we undertook a feeding study in marmoset monkeys in which co-twin males were fed during the neonatal period (the first 6 weeks of life) with either standard formula milk (SMA) or with SFM and testicular development and function was assessed at the end of this period (Sharpe et al., 2002). As marmosets exhibit a period of neonatal testicular activity akin to that in the human male (Lunn et al., 1994; Mann and Fraser, 1996; McKinnell et al., 2001), and as their infants were fed on demand in the same way that human infants are fed with formula milk, most of the issues concerning relevance of laboratory animal studies to the human were thus avoided. These studies demonstrated that feeding with SFM attenuated the neonatal testosterone rise and that this was associated, paradoxically, with a 75% increase in Leydig cell numbers at age approximately 6 weeks (Sharpe et al., 2002). There were no other significant changes in testicular cell numbers or morphology, and body weight and general growth characteristics were comparable in neonatal co-twins fed with either SMA or SFM. We have since followed some of these treated animals through puberty and into adulthood when their fertility and testicular structure and function have been assessed, together with general characteristics of the reproductive system and other organ weights. These data form the basis of the present article.

Materials and methods

Animals and treatments

Animals were captive-bred common marmoset monkeys (Callithrix jacchus), maintained in a colony that has been self-sustaining since 1973. For the present studies, a total of 14 newborn male marmosets were used, comprising seven pairs of co-twins which were presumed to be fraternal due to the high (85%) dizygotic twinning rate in this species. Marmosets show considerable between-animal variability that would normally necessitate the use of larger numbers of animals, based on power calculations, but the use of a co-twin study design (1 twin SMA-fed, 1 twin SFM-fed) enabled pairwise evaluation of data and thus minimized between-animal variability and the use of more animals (Sharpe et al., 2000, 2002, 2003). Commencing at 4–5 days of age, infant marmosets were treated as follows. During the daytime (approximately 8 h on weekdays and approximately 2 h at weekends), infant animals were separated from their mothers and left with their fathers in the family cage, though, by using a wire mesh divider, they remained within sight of their mothers. As the marmosets are maintained in family groups and it is normally the father or older siblings who look after infant marmosets (apart from feeding), this daytime separation caused no obvious distress or problems for either mothers or infants. During the period when the mother was absent from the family cage, the infant animals were removed at set intervals and hand-fed with either standard (cow’s milk-based) formula milk (‘SMA Gold’; SMA Nutrition, Taplow, Berkshire, UK) or with SFM (‘Wysoy’; SMA Nutrition) from days 4 to 5 until days 35–45; both formula milks were purchased from a supermarket. The formula powders were added to tapwater, diluted and heated as per the manufacturer’s instructions. The suspension was taken up into a 1 ml syringe with a soft silicon rubber tube fitted to the end and offered to the infant. Both SMA-fed (controls) and SFM-fed animals showed normal rates of weight gain during the feeding period (Sharpe et al., 2002).
The isoflavone content of the SFM used in the present studies was not measured, but based on analyses of various soy-based infant formulae bought in the UK, levels in the range 18–41 mg aglycone/l would be predicted and mean levels of 25.5 mg aglycone/l were reported for the particular brand of SFM that we used (Irvine et al., 1998a; Knight et al., 1998; Ministry of Agriculture, Food and Fisheries, UK, 1998; Setchell et al., 1998); isoflavones were not detected in any of the brands of cow’s formula milk that were analysed (Ministry of Agriculture, Food and Fisheries, UK, 1998). In all other respects, SFM has been constituted to provide comparable nutritional/calorific intake to cow’s milk-based infant formulae.

To monitor puberty, serum testosterone levels were measured at 10-week intervals, commencing at 40 weeks of age and continuing until 120 weeks of age. A blood sample (approximately 0.5 ml) was obtained from the femoral vein of each male using a 1 ml heparinized syringe fitted with a 27G needle, and the plasma was then separated by centrifugation and stored at –20°C until used for testosterone assay. At age 120–138 weeks, the seven sets of co-twin males were killed via the intraperitoneal injection of an overdose of sodium pentobarbitone (Euthatal; Rhone Merieux, Harlow, Essex, UK).

These studies were approved by the local ethical committee for studies in primates and were performed according to the Animal Scientific Procedures (UK) Act 1986 under Project Licence approval by the UK Home Office.

**Tissue collection and processing**

Testes with epididymides attached were dissected free of connective tissue and immersion fixed for either 5.5 h (left testis) or 24 h (right testis) in Bouins, after which each testis was dissected away from the epididymis and weighed. The prostate, seminal vesicles, thymus, spleen and pituitary gland were also dissected out and immersion fixed for 5.5 h in Bouins, after which each organ was weighed. All tissues other than the right testis were then processed for 17.5 h in an automated Leica TP-1050 processor and embedded in paraffin wax. Sections of 5 μm thickness were cut and floated onto slides coated with 2% (w/v) 3-aminopropyltriethoxy-silane (Sigma-Aldrich, Dorset, UK) and dried at 50°C overnight before being used for morphological evaluation and for cell quantification studies as described below. After fixation, the right testis was sampled in a random systematic manner, i.e. of four transverse slices either slices 1 and 3 or slices 2 and 4 were sampled. These were processed through graded ethanols before infiltration with JB4 resin (TAAB, Berkshire, UK) and used subsequently for enumeration of Sertoli cells using the optical dissector method.

**Determination of Sertoli cell number**

After polymerization of the JB4 resin, 20 μm sections were cut on a Reichart 2050 microtome using a Diatome Histoknife, mounted onto glass slides and stained with Harris’ haematoxylin. Sertoli cells were then counted using the optical dissector method as described by Wreford (1995) and as reported previously by us for the marmoset (Sharpe et al., 2000).

**Determination of Leydig cell number**

Leydig cell volume per testis was determined using paraffin sections of testis that had been immunostained for 3β-hydroxysteroid dehydrogenase (3β-HSD) as described previously (Sharpe et al., 2000). The method used Image-Pro Plus 4.5.1 with Stereologer-Pro 5 plug-in software (Media Cybernetics UK, Wokingham, Berkshire, UK) and utilized an Olympus BH-2 microscope fitted with a Prior automatic stage (Prior Scientific Instruments, Cambridge, UK). The software was used to select random fields for counting and to place a grid over the tissue. The number of fields counted per animal (approximately 60 per animal, 20 fields per section, three sections per animal) was dependent on obtaining a percentage SE value of <5%. Points falling over 3β-HSD-positive cytoplasm or over the nuclei of cells with 3β-HSD-positive cytoplasm were scored separately, and both were then independently expressed as relative volumes per testis. These data were converted to absolute volumes per testis by multiplying by testis weight (equivalent to volume), as shrinkage was minimal. These data were converted to Leydig cell number per testis after determination of mean Leydig cell nuclear diameter and volumes (average of 70–100 nuclei) using the selector function of the Stereologer-Pro 5 software, which calculates volume from an average of three nuclear diameter measurements of the same Leydig cell nucleus. Average Leydig cell cytoplasmic volume was computed by dividing Leydig cell cytoplasmic volume per testis by the number of Leydig cells.

**Plasma levels of testosterone**

Levels of testosterone in plasma were measured using an enzyme-linked immunosorbent assay adapted from an earlier radioimmunoassay method (Corker and Davidson, 1981), as detailed previously (Sharpe et al., 2002). The limit of detection was 12 pg/ml, and inter- and intra-assay coefficients of variation were <15%. Samples from each set of co-twins were always extracted and assayed at the same time.

**Statistics**

As only co-twins were used for this study, paired t-test comparison of body weight, testis weight and cell number/volume data for SMA-fed (controls) and SFM-fed animals was utilized for all such comparisons. Testosterone data was log-transformed prior to analysis because of the considerable variation between sets of twins and because of heterogeneity of variance.

**Results**

**Formula intake**

Detailed comparison of the intake of SMA and SFM by co-twins during the feeding period was reported in an earlier publication (Sharpe et al., 2002) and revealed no significant difference between the two feeding regimes. Based on the average isoflavone content of the SFM brand used for feeding, the intake of isoflavones by the SFM-fed co-twins was estimated at 1.6–3.5 mg/kg/day, which is 40–87% of that reported in human infants aged 4 months who were fed on a 100% SFM diet (Setchell et al., 1997).

**Body weight and other organ weights**

All co-twins in the present study grew normally, and when killed in adulthood the body weights of SFM– and SMA–fed co-twins were comparable (Table I). Similarly, weights of the prostate, seminal vesicles and pituitary gland were all comparable in SFM– and SMA–fed co-twins, as was stretched penis length (Table I). As earlier rodent studies had indicated that
genistein might have adverse effects on thymus weight and the immune system (see Introduction), the weights of the thymus and spleen were also measured in SMA- and SFM-fed co-twins and were found to be similar (Table I). No obvious abnormalities of other organ systems in the two sets of co-twins were observed at autopsy (data not shown).

**Onset and progression of puberty**

The onset of puberty was gauged by measurement of serum testosterone levels at 10- to 20-week intervals. Judged by the age at which testosterone levels first increased above baseline levels (>0.5 mg/ml), puberty was initiated between 40 and 60 weeks of age in SMA- and SFM-fed co-twins, with no consistent difference in timing between the two feeding groups (Figure 1). Thereafter, mean serum testosterone levels increased gradually up to fully adult levels, which were attained between 70 and 120 weeks of age (Figure 1). Because of the episodic nature of testosterone secretion, testosterone levels in individual animals and between individuals varied considerably for both sets of twins at all of the ages sampled. There was no significant difference in mean serum testosterone levels between SMA- and SFM-fed co-twins at any of the ages, although two of the SFM-fed twins (numbers 1 and 6) at 90 days of age and beyond consistently had testosterone levels within the lower end (0.7–6.3 ng/ml) of the normal range.

![Figure 1. Onset and progression of puberty in co-twin marmoset monkeys fed as infants with standard formula milk (SMA) or with soy formula milk (SFM), as monitored by serum levels of testosterone. The data show means ± SEM for seven sets of co-twins at each age.](image)

**Fertility**

At age 80–104 weeks, each male was placed with an adult female marmoset and maintained in cohabitation for a period of 24–80 weeks until the end of the study. Pregnancy was confirmed in the female partners by palpation and the numbers of offspring were recorded. All SMA- and all but one of the SFM-fed co-twins (number 6) proved to be fertile, and there was no obvious difference in fertility between the two groups (Table II). SFM-fed co-twin number 6 exhibited full and normal spermatogenesis, based on testicular histology, so there was no obvious reason to suspect infertility in this animal. However, this animal consistently had low-normal testosterone levels in adulthood (averaging 2.0 ng/ml for the period 90–120 days; see Figure 1 for comparison with group mean values). Nevertheless, as fertility in the co-habiting female was unproven, it cannot be excluded that she was infertile. Although no formal assessment of sexual and mating behaviour was undertaken, there were no apparent signs of differences between the two groups of males.

**Testicular morphology, weight and cell numbers**

Average testicular weight in SFM-fed co-twins was 14% heavier than in SMA-fed control co-twins, a difference that achieved statistically significance (P = 0.041, Figure 2). Comparison of testicular weights in individual co-twins revealed that in all but one set of twins, testis weight in SFM-fed animals was always greater than testis weight in the control co-twin, this difference ranging from 7 to 32% (Figure 2). The one exception was for co-twins number 7, in which testis weight in the SFM-fed co-twin was slightly lower than that in the SMA-fed control co-twin (Figure 2), but it was discovered upon histological assessment of testicular sections that the SFM-fed co-twin number 7 exhibited quite a pronounced incidence of Sertoli cell-only (SCO) seminiferous tubules, possibly as a consequence of partial blockage of the efferent ducts or rete. As the loss of germ cells from the SCO tubules would have resulted in a reduction in testis weight, it is

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SMA-fed males</th>
<th>SFM-fed males</th>
<th>SFM as percentage of SMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>407 ± 54</td>
<td>420 ± 26</td>
<td>103</td>
</tr>
<tr>
<td>Prostate weight (mg)</td>
<td>275 ± 72</td>
<td>238 ± 72</td>
<td>87</td>
</tr>
<tr>
<td>Seminal vesicle weight (mg)</td>
<td>279 ± 149</td>
<td>242 ± 50</td>
<td>87</td>
</tr>
<tr>
<td>Penile weight (mg)</td>
<td>11.9 ± 3.6</td>
<td>12.4 ± 1.9</td>
<td>104</td>
</tr>
<tr>
<td>Spleen weight (mg)</td>
<td>385 ± 94</td>
<td>370 ± 182</td>
<td>96</td>
</tr>
<tr>
<td>Thymus weight (mg)</td>
<td>387 ± 556</td>
<td>312 ± 94</td>
<td>81</td>
</tr>
<tr>
<td>Penis length (mm)</td>
<td>17.0 ± 1.6</td>
<td>17.8 ± 1.2</td>
<td>105</td>
</tr>
</tbody>
</table>

n = 7 per group.

*One SMA-fed male had an exceptionally large thymus (1153 mg); exclusion of this value resulted in a substantially lower mean ± SD value for the SMA group (260 ± 124).

**Table I.** Mean ± SD body and organ weights and penis length in adult co-twin marmosets fed as infants with standard formula milk (SMA-fed) or with soy formula milk (SFM-fed).

<table>
<thead>
<tr>
<th>Co-twin number</th>
<th>Formula type</th>
<th>Months of cohabitation with female</th>
<th>Number of pregnancies</th>
<th>Offspring number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SMA</td>
<td>6</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>SMA</td>
<td>6</td>
<td>2</td>
<td>2 + 2</td>
</tr>
<tr>
<td>3</td>
<td>SFM</td>
<td>20</td>
<td>2</td>
<td>2 + 1 + 1</td>
</tr>
<tr>
<td>4</td>
<td>SFM</td>
<td>11</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>SMA</td>
<td>11</td>
<td>3</td>
<td>2 + 2 + 1 + 3</td>
</tr>
<tr>
<td>6</td>
<td>SMA</td>
<td>11</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>SFM</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>SFM</td>
<td>6</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

*For animal husbandry reasons, the female partner was placed on a hormonal contraceptive regime (Depo-provera) after birth of the first offspring, so no further pregnancies were possible in these cases.

*Fertility of the cohabiting female was unproven.
tentatively concluded that this animal would also have exhibited a higher testis weight than its control co-twin if it had not had SCO tubules. Apart from this particular animal, testicular morphology appeared normal and comparable in all other SMA- and SFM-fed co-twins (Figure 3), and, as SCO tubules were not found in any other SFM-fed animal, the occurrence of these tubules in co-twin number 7 is most likely sporadic rather than treatment related.

Sertoli cell number, measured using the optical dissector, revealed an overall 7% increase in SFM- compared with SMA-fed co-twins \( (P = 0.025) \), and comparison of individual co-twins revealed that Sertoli cell number was higher in the SFM-fed co-twin in every instance (Figure 4). A similar comparison of Leydig cell number revealed an average 32% higher number of Leydig cells in SFM-fed co-twins than was found in their SMA-fed co-twin controls \( (P = 0.026, \text{Figure 4}) \), though this assessment was based only on six sets of co-twins for technical reasons. As was found with Sertoli cell number, comparison of Leydig cell number in individual sets of co-twins revealed a higher number of Leydig cells in the SFM-fed co-twin in each instance, although there was considerable variation in the magnitude of this difference between individual sets of co-twins (Figure 4). In this regard, it was notable that the two SFM-fed co-twins (numbers 1 and 6) with the largest increase in Leydig cell number compared with their control co-twin (Figure 4) were also the animals that had consistently low-normal testosterone levels in adulthood, as mentioned above. Average Leydig cell cytoplasmic volume was slightly, but non-significantly smaller in SFM-fed compared with SMA-fed males, but no consistent trend of SFM-fed twins displaying smaller Leydig cell cytoplasmic volumes than their SMA-fed co-twins was observed (data not shown).

Mean numbers of germ cells per testis were not assessed in this study due to major differences in the shape and average diameter of the nuclei of the different germ cell types present in the seminiferous epithelium. However, stereology was used to provide a measure of the average volume of the seminiferous epithelium (which is composed predominantly of germ cells) per tubule. This revealed a slightly higher mean value for seminiferous epithelial volume in SFM-fed males, but there was no consistent trend in the difference between SMA- and SFM-fed co-twins (Figure 5).

**Discussion**

The primary objective of the present study was to follow-up our earlier study (Sharpe et al., 2002) of the effects of infant

![Figure 2. Testicular weight in adulthood in co-twin marmosets fed as infants with standard formula milk (SMA) or with soy formula milk (SFM). Data are shown individually for the seven sets of co-twin marmosets and mean data are shown to the right. Statistical significance was determined using the paired t-test.](image)

![Figure 3. Comparative testicular histology in two adult co-twin marmosets fed as infants with standard formula milk (SMA) or with soy formula milk (SFM). Leydig cell cytoplasm has been immunostained for 3β-hydroxysteroid dehydrogenase (3β-HSD) (brown). Scale bar shows 100 μm.](image)
feeding with SFM in the marmoset on reproductive function in adulthood. The overall motivation for the earlier and present studies was to address the concerns that have been expressed about potential adverse health effects of feeding infants with SFM, in particular because of its high phytoestrogen content (Badger et al., 2002; UK Working Group on Phytoestrogens and Health, 2003; Chen and Rogan, 2004). By using a non-human primate and a co-twin male design, and by using oral feeding analogous to that which occurs in human infants, our studies have largely bypassed the problems associated with administration of SFM or its constituent compounds to laboratory animals, in which the mode of administration and timing of development are not comparable to that of the human (see Introduction). As our earlier findings had shown that feeding with SFM could partially suppress the neonatal testosterone rise in male marmosets, there was concern as to whether this ‘adverse’ effect might have long-term consequences in terms of reproductive function in adulthood (Sharpe et al., 2002). The present findings are generally reassuring in this respect, in that they show no obvious major effect on the timing or progression of puberty or on fertility of most SFM-fed males in adulthood. Our findings are also encouraging with regard to the development of other reproductive organs and to the function of the immune system, at least insofar as weights of the thymus and the spleen are able to indicate this. Unexpectedly, our findings show that testicular weight and somatic cell numbers are significantly increased in animals that have been fed with SFM as infants. The latter changes did not result in any values in SFM-fed males that lay outside of the control range of values, and these differences would not have been detectable if the present study had not used a paired, co-twin design. However, there is some concern that these changes may reflect a degree of ‘compensated Leydig cell failure’, which merits further investigation.

Figure 4. Number of Leydig (3β-HSD-immunopositive) (top) and Sertoli cells (bottom) per testis in adult co-twin marmosets fed as infants with standard formula milk (SMA) or with soy formula milk (SFM). Data are shown individually for the seven sets of co-twin marmosets and mean data are shown to the right. Note that SFM-fed co-twins 1 and 6 consistently had serum testosterone levels within the lower end of the normal range in adulthood (90–120 days of age). Statistical significance was determined using the paired t-test. Note that due to technical reasons, it was not possible to determine Leydig cell number for co-twins number 3.

Figure 5. Mean seminiferous epithelium volume per tubule in adult co-twin marmosets fed as infants with standard formula milk (SMA) or with soy formula milk (SFM). Data are shown individually for the seven sets of co-twin marmosets and mean data are shown to the right. Statistical significance was determined using the paired t-test.

Figure 6. Mean lumen volume per seminiferous tubule in adult co-twin marmosets fed as infants with standard formula milk (SMA) or with soy formula milk (SFM). Data are shown individually for the seven sets of co-twin marmosets and mean data are shown to the right. Statistical significance was determined using the paired t-test.
It is likely that the present findings in marmosets are directly relevant to the concerns about the human health effects of infant feeding with SFM or with exposure to soy or its constituents in various other foods during infancy, although this assumes similar uptake and metabolism of the SFM components in infant marmosets and humans. Our choice of using the marmoset as a model for the human was based on the comparability of the phases of male reproductive development in these two species (Mann and Fraser, 1996; McKinnell et al., 2001), together with the ability to use a co-twin design, which minimized the use of animals. This also allowed a more sophisticated evaluation of potentially subtle effects by using non-identical male twins, which experience has shown are far more comparable for various reproductive parameters than are unrelated males of the same age (Sharpe et al., 2000, 2002, 2003). The fact that we were able to administer SFM to the marmosets in a manner analogous to that in formula-fed human infants (Sharpe et al., 2002) was also a key factor in our studies. Our infants were fed on demand at regular intervals during the day, though for technical reasons (manpower limitations and animal welfare concerns), we were unable to use 100% feeding with SFM and therefore only achieved intakes of SFM that amounted to 40–87% (Sharpe et al., 2002) of that reported in human infants at age 4 months who were fed solely with SFM (Setchell et al., 1997). Based on these comparisons, it is logical to presume that similar effects will be observed in human infants fed with SFM as those that are described presently in SFM-fed marmosets; it is also likely that any such effects may be of greater magnitude if the human infants have been fed solely with SFM. This being the case, the issue is then whether such effects might be considered adverse and/or have health implications, either beneficial or adverse.

In the present study, SFM feeding of infant marmosets, which we had previously shown to attenuate the neonatal testosterone rise (Sharpe et al., 2002), had no obvious or significant effect on the timing or progression of puberty, on fertility in adulthood or on penis development and length. These findings are largely consistent with earlier studies in marmosets, and in other non-human primates, in which total ablation of the neonatal testosterone rise, via administration of a GnRH antagonist, had either no effect (fertility) or relatively minor effects (slightly retarded puberty and penis growth) (Mann et al., 1993; Lunn et al., 1994, 1997; Mann and Fraser, 1996). Nevertheless, it must be kept in mind that the present study was based only on seven sets of twins and is therefore not powered to detect low prevalence effects. In this regard, we cannot exclude that infant feeding with SFM can impair fertility in some males. Similarly, although SFM feeding of marmosets also had no effect on the weights of the seminal vesicles or prostate, it would be prudent to evaluate prostatic size in older men who were reared as infants on SFM, in view of the well-described effects of perinatal estrogen exposure on prostate development (Harkonen and Makela, 2004), and the long-term effects of neonatal genistein exposure on uterine adenocarcinoma in rodents (Newbold et al., 2001). Detailed analysis of prostatic histology in SFM-fed co-twins from the present study may also be instructive in this regard.

The most significant, and unexpected, of the present findings were the significant increases in testicular weight and numbers of Sertoli cells and Leydig cells in the testes of marmosets fed with SFM as opposed to SMA in infancy. At face value, these increases are not obviously ‘adverse’, though the fact that infant exposure to SFM has induced permanent changes to the cell composition of the testis does raise the possibility of effects in other organ systems that might be adverse. There is also concern that the increase in Leydig cell numbers in SFM-fed co-twins in the presence of normal serum testosterone levels could be indicative of some degree of ‘compensated Leydig cell failure’ (de Kretser, 2004). The finding that two of the SFM-fed marmosets had testosterone levels in adult samples (90–120 days of age) that were consistently towards the lower end of the normal range supports such a view, especially as these two animals showed by far the largest increases (214% increase in co-twin 1 and 55% increase in co-twin 6) in Leydig cells per testis in comparison with their co-twin controls. The fact that Leydig cell number was increased to a lesser extent in other SFM-fed males would be consistent with a milder degree of compensated Leydig cell failure in these animals. One sign of compensated Leydig cell failure can be the presence of supranormal serum levels of LH (de Kretser, 2004), but unfortunately it was not possible to assess this in the present study as there is still no available serum LH assay for marmosets. In this regard, it may prove useful to monitor serum levels of LH and testosterone in groups of young adult men who have been reared as infants on SFM or on other formula milk to establish whether or not there is a normal LH : testosterone ratio.

Irrespective of the aetiology of the increase in Leydig cell numbers in SFM-fed co-twin marmosets, this finding is consistent with our earlier observation in marmosets that complete suppression of the neonatal testosterone rise in infancy, via administration of a GnRH antagonist, results in increased Leydig cell number/volume per testis in adulthood (Sharpe et al., 2000). The mechanisms underlying such changes and the somewhat smaller (but consistent and significant) increase in Sertoli cell numbers in SFM-fed co-twin males in the present study are unclear, but elevation of gonadotrophin levels (both LH and FSH) could provide a logical explanation. These observations add to the growing evidence that events or exposures in perinatal life can have important consequences for testicular structure and/or function in adulthood, especially when effects on reproductive hormones are involved (Sharpe et al., 2000, 2003; Skakkebaek et al., 2001; Sharpe and Irvine, 2004).

The increase in testicular weight in SFM-fed marmosets in the present study could be a consequence of the general increase in cell numbers per testis or an increase in fluid content of the testis; the latter possibility arises because of the well-established effects of estrogens on fluid resorption from the excurrent duct system of the testis (Hess et al., 2001), and exposure to SFM during infancy would have resulted in supranormal estrogen exposure via the component phytoestrogens (Setchell et al., 1997, 1998; Irvine et al., 1998a,b). The possibility of any major such effects was excluded in the present studies based on analysis of seminiferous tubule lumen volume per tubule, which was shown to be similar in SFM-fed and SMA-fed co-twins. Although indirect evidence of disruption of the excurrent duct
system was observed in one SFM-fed marmoset (number 7) and was shown to result in increased lumen volume per testis and the occurrence of SCO tubules, the absence of any such findings in the other six SFM-fed marmosets suggests that the findings in this particular animal most likely have another cause that is unrelated to the method of feeding in infancy.

It is therefore more likely that the increase in testicular weight in SFM-fed marmosets is a result of an increase in cell numbers per testis. The finding of higher numbers of Leydig and Sertoli cells per testis in SFM-fed animals is therefore consistent with this interpretation, as is the finding of a less consistent increase in the volume of seminiferous epithelium per tubule in the same animals, as the latter reflects mainly germ cell volume, which is the main determinant of testis size in an adult male. Because we determined seminiferous epithelial volume per tubule in the present study, it was not strictly legitimate to convert this to a volume per testis by multiplying by testis weight. However, if such a correction was applied, it revealed an average 26% increase in seminiferous epithelial volume per testis in SFM-fed compared with SMA-fed males, suggesting that increase in germ cell volume per testis is the most likely explanation for the increase in testis weight in SFM-fed versus SMA-fed males. It is likely that this increase is in turn a consequence of the increase in Sertoli cell numbers in SFM-fed males, as Sertoli cell number is the ultimate determinant of germ cell volume per testis and testis size in adult males (Sharpe, 1994).

In experimental studies in various animals, most have reported no effects of perinatal phytoestrogen exposure on adult testis weight (Badger et al., 2001; Delclos et al., 2001; Nagao et al., 2001; Fritz et al., 2003; Masutomi et al., 2003; Chen and Rogan, 2004; Jung et al., 2004), although one study in mink reported a significant increase in testis weight (Ryokkynen et al., 2005). Though some studies have reported significant adverse effects on reproductive function in male rodents after perinatal phytoestrogen exposure (Delclos et al., 2001; Wisniewski et al., 2003), such observations have generally only been made after the administration of very high amounts of compounds, and it is well established in laboratory animals that perinatal exposure to high levels of estrogens can induce such abnormalities (Atanassova et al., 2000; Fritz et al., 2003). It will be important in human studies to test whether similar changes in testis size to those reported presently in the marmoset can be detected in adult men who were fed SFM as infants. If such studies are to be undertaken, they will have to use large numbers of subjects, because adult testicular size is extremely variable in normal men and will have to use an accurate means of assessing testicular size such as ultrasound (Behre et al., 1989; Carlsen et al., 2000). It will not be possible in such men to evaluate whether or not there is any increase in Sertoli or Leydig cell numbers, as found in the present studies in SFM-fed marmosets, as this would require removal or biopsy of the testis.

In conclusion, the present studies show that infant feeding with SFM in primates does not have dramatically adverse reproductive consequences in the male in adulthood, such as have been reported in some rodent studies. The main caveat to this conclusion is the possibility that there is consistent but variable degrees of compensated Leydig cell failure in the SFM-fed males, although this is based largely on indirect evidence.

This can probably be evaluated in human subjects by determination of the LH : testosterone ratio in adult men who were fed as infants with SFM and compared with appropriate controls. Even though the present findings are generally reassuring, they do not rule out the possibility that infant feeding with SFM might induce adverse effects in a small proportion of exposed males, as our study was based only on small numbers of animals. In this regard, our findings reaffirm that any intervention in perinatal life that involves altered hormone exposure of the infant is likely to have adult consequences, as is demonstrated in the present studies. As such consequences may not always be favourable or benign, it seems prudent to recommend that any hormonal exposures of the infant male, especially during the period of the neonatal testosterone rise (0–6 months of age), should be avoided whenever possible.

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References

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