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Reproduction, Fertility and Development

Maternal high-fat diet during pregnancy and lactation affects factors that regulate cell proliferation and apoptosis in the testis of adult progeny

Helen Viotti[A], Daniel Cavestany[A], Graeme B. Martin[B,*], Mark H. Vickers[C], Deborah M. Sloboda[D,E,F,G] and Graciela Pedrana[A]

For full list of author affiliations and declarations see end of paper

***Correspondence to:**
Graeme B. Martin
UWA School of Agriculture and Environment, and UWA Institute of Agriculture, University of Western Australia, Perth, WA, Australia
Email: Graeme.Martin@uwa.edu.au

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ABSTRACT

Context. A maternal high-fat diet is thought to pose a risk to spermatogenesis in the progeny. **Aims.** We tested whether a maternal high-fat diet would affect Sertoli cell expression of transcription factors (insulin-like growth factor I (IGF-I); glial-cell line-derived neurotrophic factor (GDNF); Ets variant 5 (ETV5)) and cell proliferation and apoptotic proteins, in the testis of adult offspring. **Methods.** Pregnant rats were fed *ad libitum* with a standard diet (Control) or a high-fat diet (HFat) throughout pregnancy and lactation. After weaning, male pups were fed the standard diet until postnatal day 160. Males were monitored daily from postnatal day 34 to determine onset of puberty. On postnatal day 160, their testes were processed for morphometry and immunohistochemistry. **Key results.** The HFat diet increased seminiferous-tubule diameter ($P < 0.03$), the numbers of Sertoli cells ($P < 0.0001$) and Ki-67-positive spermatogonia ($P < 0.0006$), and the areas immunostained for ETV5 ($P < 0.0001$), caspase-3 ($P < 0.001$) and Bcl-2 ($P < 0.0001$). By contrast, the HFat diet reduced the areas immunostained for IGF-I ($P < 0.01$) and GDNF ($P < 0.0001$). **Conclusions.** A maternal high-fat diet alters the balance between spermatogonia proliferation and spermatid apoptosis. **Implications.** A maternal high-fat diet seems to 'program' adult male fertility.

Keywords: apoptosis, cell proliferation, developmental programming, high-fat diet, rat, reproduction, Sertoli cell, spermatogenesis, testis.

Introduction

The 'developmental programming' hypothesis suggests that the maternal environment during prenatal or early postnatal life influences the subsequent health and performance of the progeny, including susceptibility to the development of obesity and related metabolic disorders. An adverse maternal environment during fetal life would also be expected to alter germ cell production and thus compromise the fertility of the next generation (Vautier and Cadaret 2022). Indeed, recent studies in the rat by our group (Pedrana *et al.* 2020, 2021) have shown that maternal undernutrition significantly impacts on several molecular factors that control testis function in developing male offspring.

Among those factors, the Sertoli cells produce insulin-like growth factor I (IGF-I) (Escott *et al.* 2014) and two important transcription factors, transcription variant 5 (ETV5) and glial-cell line-derived neurotrophic factor (GDNF) that are essential to achieve a testicular microenvironment and the development of normal spermatogenesis (Yang and Han 2010; Alves *et al.* 2016). These proteins that act upon receptors on spermatogonial stem cells (SSCs), in turn induce critical genes for SSC self-renewal. Particularly, ETV5 acts also in an autocrine manner to contribute to the production of Sertoli chemoattraction factors for germ cells (Eo *et al.* 2012) and induce spermatogonial cell proliferation of ETV5 (Schlesser *et al.* 2008).

Furthermore, apoptotic proteins, cysteinyl-aspartate protease 3 (caspase-3), Bcl-2 associated X protein (Bax) and B-cell lymphoma-2 (Bcl-2) determine the balance between apoptosis and proliferation, which determines sperm output. The apoptosis process is a strategy to limit the number of germ cells that a Sertoli cell must support, and Sertoli cells themselves control germ cell apoptosis through paracrine signalling that balances pro-apoptotic factors (caspase-3, Bax) and anti-apoptotic factors such as Bcl-2 (Murphy and Richburg 2014).

The effects of maternal diets on molecular and cellular programming in the testes of offspring are probably not confined to maternal undernutrition. For example, we have shown previously that a maternal obesogenic diet increases the risk of obesity and a variety of cardiometabolic disorders, including postnatal hyperglycemia, hyperinsulinemia and dyslipidemia, in male offspring (Sloboda *et al.* 2009; Howie *et al.* 2013; Langley-Evans 2015). Moreover, a maternal high-fat diet can impair the testicular antioxidant defence system, thus increasing oxidative stress (Bautista *et al.* 2017; Billah *et al.* 2022), and suppress apoptosis, reduce sperm count and impair sperm function (Ruiz Valderrama *et al.* 2016; Mao *et al.* 2018). Many of these factors are associated with premature aging of the reproductive system and poor fertility (Williams *et al.* 2014; Bautista *et al.* 2017). In female offspring, there are comparable consequences for ovarian function (Tsoulis *et al.* 2016).

Despite the fact that we have made significant advances in our understanding of testicular development after prenatal dietary manipulation, we still do not have a complete understanding of the mechanisms that underlie the effects of a maternal high-fat diet on testis development in the progeny. One of the key points to consider is the balance between cell proliferation and apoptosis in the developing testis because it is a critical aspect of the regulation of spermatogenesis and determination of sperm output, and thus probably 'programs' future fertility. Therefore, in the present study, we focused on how a maternal obesogenic diet affects: (1) the pro- and anti-apoptotic proteins in germ cells (Shaha *et al.* 2010; Rodrigo *et al.* 2022); (2) the Sertoli cell-produced transcription factors that regulate self-renewal of spermatogonial stem cells (Parekh *et al.* 2019; Zhang *et al.* 2021); and (3) IGF-I, also produced by Sertoli cells as an autocrine regulator of spermatogonial renewal, cell growth, proliferation and differentiation (Griffeth *et al.* 2014). In our rat model, we used immunoexpression to test whether a maternal high-fat diet during pregnancy and lactation would increase the Sertoli cell GDNF and ETV5, decrease active caspase-3 and Bax, increase Bcl-2, and decrease IGF-I in the testis of post-pubertal progeny. We also measured plasma concentrations of androgens because maternal obesity during pregnancy and lactation leads to Leydig cell atrophy and impaired steroidogenesis in pups when they reach adulthood (Pinto-Fochi *et al.* 2016).

Materials and methods

Animal model

All animal procedures were performed under the guidelines of the Animal Ethics Committee of the University of Auckland (R402). An established model of developmental programming based on manipulation of maternal nutrition was used (Howie *et al.* 2012). Female Wistar rats (120 days old; $n = 10$) from the Vernon Jansen Unit at the University of Auckland were time-mated using a rat oestrous cycle monitor (Fine Science Tools, USA). Thereafter, they were housed individually in standard rat cages in the same room, with free access to water, a constant temperature of 25°C and a 12:12 light: dark cycle. Pregnant dams were randomly allocated to two dietary treatments (five per group): (1) Control – dams fed *ad libitum* throughout pregnancy and lactation with a standard diet (fat 6.2%, protein 18.6%; carbohydrate 44.2%; Teklad Global 18% Protein Diet, Diet 2018, calories (kcals/g) from fat 18%, from protein 24%, calories from carbohydrate 58%, https://insights.envigo.com/hubfs/resources/data-sheets/2018-datasheet-0915.pdf); and (2) HFat – dams were fed a high-fat diet throughout pregnancy and lactation (fat 24%, protein 24%, carbohydrate 41%; calories (kcals/g) from fat 45%, calories from protein 20%, calories from carbohydrate 35%, High fat D12451, Research Diets, Inc New Brunswick, NJ, USA, http://67.20.83.195/china/pdf/Data%20Sheets/D12451.pdf). After birth, pups were weighed, and litters were adjusted to comprise four males plus four females to ensure standardised nutrition until weaning (day 22). After weaning, male pups were housed in pairs and fed the standard chow diet *ad libitum* until the end of the study (day 160). Offspring body weight were recorded at birth, early post-puberty (day 42) and at the end of the trial on postnatal day 160.

Testis sampling and histological processing

On postnatal day 160, male pups ($n = 20$; 10 per dietary group) were fasted overnight, weighed, anaesthetised with pentobarbital (60 mg kg^{-1} i.p.) and then killed by decapitation. Blood was collected into heparinised tubes, and plasma was separated by centrifugation (2400*g*, 20 min) and stored at −20°C until later analysis. Testes were weighed and the data were used to calculate relative testis mass (g testis mass per g body mass) and thus the gonadosomatic index (relative testis mass × 100). Left testes were frozen in liquid nitrogen and stored at −80°C for molecular analyses. Right testes were immersed in fixative (4% formaldehyde phosphate buffer, pH 7.4) for 24 h for histology and immunohistochemistry (Fig. 1).

Fixed testes were progressively dehydrated by immersion in increasing concentrations of ethanol (70%, 95%, 100%), embedded in paraffin resin (Historesin; Leica, Germany) and sectioned at 5 μm by microtome (Leica 149 Reichert

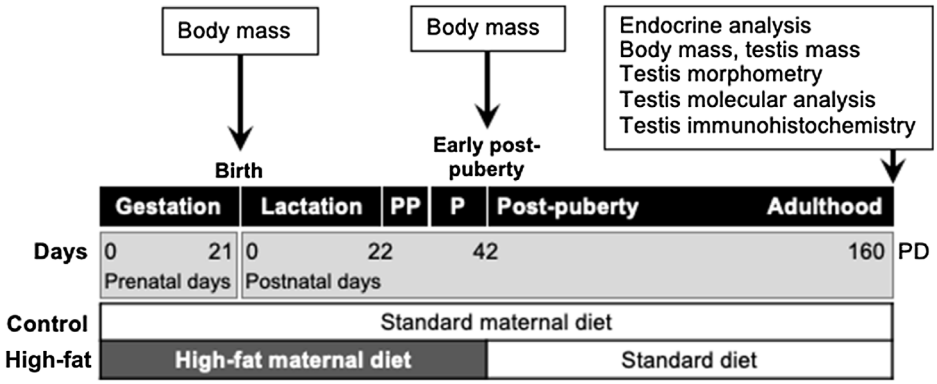

Fig. 1. Schematic representation of the experimental design. There were two treatments for maternal nutrition (Control and High-fat) for gestation days (GD) 0–21, and for lactation, from birth (prenatal day 21) to weaning on postnatal day (PD) 22. After weaning, male pups were fed *ad libitum* with a control diet until the end of the study (PD 160) when blood and testes were sampled. Puberty (P) was observed over the period 35-37 PD. PP, pre-puberty.

Jung Biocut 2030, Wetzlar, Germany) in preparation for hematoxylin–eosin staining and immunohistochemistry.

Endocrine assays

Commercially available assays from Diagnostic Systems Laboratories Inc. (Webster, TX, USA) were used to measure the plasma concentrations of testosterone (DSL-4100) and androstenedione (DSL-4200). We followed the manufacturer's instructions and included all samples within a single assay. For testosterone, the limit of detection was 0.10 ng/mL and the within-assay coefficient of variation (WACV) was 2.8%. For androstenedione, the limit of detection was 0.10 ng/mL and the WACV was 5.7%.

Immunohistochemistry

Slides were processed for the detection by immunoexpression of ETV5, GDNF, IGF-I, caspase-3 Bax, Bcl-2 and Ki-67 using the streptavidin–biotin–peroxidase protocol, as described previously (Pedrana *et al.* 2020). In brief: the tissue sections were immersed in 250 mL of 0.01 M citrate buffer solution (pH 6.0) with Tween-20 (5 mL) at a concentration of 2% for dewaxing, hydration and epitope retrieval by heating for 5 min in microwave oven (100% power); slides were then washed with distilled water and phosphate-buffered saline (PBS, pH 7.4); endogenous peroxidases were inactivated by treatment with 3% hydrogen peroxide 200 (20 min), followed by washing in PBS; non-specific binding proteins were blocked by incubation with normal rat serum; each slide was then incubated for 30 min with a different primary antibody (Table 1), for 30 min with biotinylated secondary antibody (rabbit-specific HRP/DAB; ab 64261; Abcam, USA), and for 30 min with streptavidin–peroxidase enzyme complex; to visualize the antigen, slides were incubated for 5 min with diaminobenzidine (DAB) chromogen solution (1 mL hydrogen peroxide + 30 µL DAB); sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted with Entellan® (Merck) on glass coverslips. All assays contained a negative control for the immunohistochemistry using different testicular sections incubated with rat serum diluted 1:100 in PBS.

Morphometry, immunohistochemistry and image analysis

Following hematoxylin-eosin staining, seminiferous tubule diameters were measured in 50 transverse cross-sections of

Table 1. Primary antibodies used in the immunohistochemical analysis of rat testis.

Target protein	Antibody	Clonality	Catalogue number, supplier	Concentration
ETV5	Anti-ETV5	Polyclonal IgG	Ab 102010, Abcam, USA	1:100
GDNF	Anti-glial cell line-derived neurotrophic factor	Polyclonal IgG	ab18956, Abcam, USA	1:200
IGF-I	Anti-IGFI	Polyclonal IgG anti-IGF-I	ab9572, Abcam, USA	4 µg/mL
Caspase-3	Anti-active caspase-3	Polyclonal IgG	AF835 R&D Systems	5 µg/mL
Bax	Anti-Bax	Monoclonal IgG Clone E63	ab32503, Abcam, USA	1:200
Bcl-2	Anti-Bcl-2	Polyclonal IgG	ab7973, Abcam, USA	1:200
Ki-67	Anti-Ki-67 antibody	Monoclonal SP6 IgG	ab16667, Abcam, USA	1:200

All antibodies were raised in the rabbit.

seminiferous tubules at a final magnification of 100×, and Sertoli cells were counted in each of 50 transverse cross-sections of seminiferous tubules at a final magnification of 400×. Digital images were retrieved using a light microscope (Olympus CX23, Olympus Scientific Solutions Americas Corp., MA, USA) connected to a digital camera (Dino-Eyepiece Edge, AM-7025X) and then captured with DinoCapture 2.0 software (https://www.dinolite.us/es/features/dinocapture/).

Immunohistochemistry images showing the immunostained area (IA) for ETV5, GDNF, IGF-I, caspase-3, Bax, Bcl-2 and the number of Ki-67-positive spermatogonial cells per transverse section of seminiferous tubules were analysed using ImageJ software (Ver. 1.52b 6 May 2018; Wayne Rasband, National Institutes of Health, USA; https://imagej.net/ij/index.html). Immunostained areas were measured using a locally written macro that included colour threshold values from processing RGB images and conversion to binary images. The threshold values were verified and normalised with controls carried across several runs for calibration. This process provided quantitative values for percentage (%) IA in 1000 digital images per testis, per animal and per immunohistochemically stained factor.

Gene expression analysis by qPCR

Total RNA was extracted using commercially available kits (AllPrep DNA/RNA mini kit; cat 80204; Qiagen, Germantown, MD, USA). Genomic DNA was removed from each sample by treatment with RNase-free DNase (Invitrogen Life Technologies, New Zealand) according to the manufacturer's instructions. RNA quantity and purity were analysed using a NanoDrop spectrophotometer (ND-1000; BioLab Ltd) and NanoDrop software (ver. 3.1.2). All RNA samples were stored at −80°C until required. For first-strand cDNA synthesis, we used 5 mg total RNA, Moloney Murine Leukaemia Virus Reverse Transcriptase (MMLV- RT; Promega Corp., WI, USA) and a standard thermocycler (GeneAmp PCR System 9700; Applied Biosystems). A master mix was prepared containing 5 mL M-MLV 5x (cat M531A; In Vitro Technologies, Madison, WI, USA), 0.5 mL M-MLV-RT (cat. M170B; In Vitro Technologies) and 1.25 mL 10 mM deoxynucleoside-triphosphates (cat. R0181, Thermo Fisher Scientific, USA). The cycling conditions were: initial denaturation for 5 min at 96°C followed by 30 cycles (30 s each) at 96°C (denaturation), 60°C (annealing) and 72°C (extension). The cDNAwas stored at −20°C until assay by quantitative polymerase chain reaction (qPCR). Testicular gene expression for caspase-3, Bax and Bcl-2, as well as the endogenous reference (b-actin), was measured by qPCR using the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, New Zealand). All primers were designed using Primer 3 software (Primer3, 0.4.0, Whitehead Institute for Biomedical Research, https://bioinfo.ut.ee/primer3-0.4.0/) and manufactured by Invitrogen Life Technologies. Primer set controls were b-actin (Table 2). Optimal primer conditions were adjusted to the cycling conditions: length, 20 bp (range 17–23 bp); temperature, 63°C (range 60–65°C); and amplicon length, 100–300 bp. Dissociation analyses were used to ensure specificity and only samples producing a single peak in the dissociation curves were retained. Amplified products were visualised on an agarose gel using the E-Gel CloneWell 0.8% SYBR Safe gel (cat. G6618-08; Invitrogen, Burlington, ON, Canada), run on the E-Gel iBase™ Power System (cat. G6400; Invitrogen, CA, USA) and sequenced by spectrophotometry (Allan Wilson Centre, Massey University, New Zealand). The resulting sequences were evaluated using NCBI BLAST to ensure specificity. Transcript levels were quantified by qPCR under the following conditions: an initial 2-min hold period at 50°C for normalisation (Stage 1), followed by enzyme activation at 95°C for 2 min (Stage 2); amplification of the gene product through 40 successive cycles of 95°C for 15 s and then 60°C for 1 min (Stage 3); a dissociation stage of 15 s at 95°C, 15 s at 60°C, and 2 min at 99°C (Stage 4). A standard curve was generated from the mean cycle threshold (Ct) of eight standards (1:5 serial dilution) of a known concentration in triplicate and amplification and dissociation curves were generated for all standards and samples (Applied Biosystems, CA, USA). Each sample was run in triplicate.

Statistical analysis

The mother was used as the biological replicate. The pups were derived from the various litters, and they were compared statistically, not the mothers. With 10 litters and 2 males per litter, we obtained 10 males per dietary group (two males from each of five litters). Although the numbers of Sertoli cells and germ cells are ordinal and discrete, we treated them as continuous variables. The data were checked for normal distribution and met the criteria for parametric testing. For each dependent variable, differences between Control and HFat were compared using two-sample

Table 2. Primers used for assessing gene expression by quantitative polymerase chain reaction in rat testis.

Rat mRNA	Forward primer	Reverse primer	Amplicon length (bp)	NCBI reference sequence
Caspase-3	GAGCTTGGAACGCGAAGAAA	TCCACGGAGGTTTCGTTGT	59	NM_012922.2
Bax	TGACGGCAACTTCAACTGGG	GCAGCCGATCTCGAAGGAA	143	NM_017059.2
Bcl-2	GGATCCAGGATAACGGAGGC	ATGCACCCAGAGTGATGCAG	141	NM_016993.1
Beta-actin	CACCAACTGGGACGATATGGA	CAGCCTGGATGGCTACGTACAT	188	NM_031144

Student's *t*-tests, aided by PROC TTEST and SAS statistical analysis (SAS, v. 9.1, SAS Institute Inc., Cary, NC, USA). The level of significance was $P < 0.05$. All data are expressed as mean ± standard error of mean (s.e.m.).

Results

Treatment had no significant effect on litter size (13.9 pups per litter in the Control vs 14.0 pups per litter in the HFat group) or on the male:female ratio (1:1.013 in the Control vs 1:1.050 in the HFat group).

Physiological parameters

The data are summarised in Table 3. There were no differences between treatments in plasma concentrations of androstenedione and testosterone. Compared to the Control group, the HFat group showed a decrease in birth weight, an earlier onset of puberty and a decrease in body weight at puberty. However, as adults at age 160 days, HFat animals were heavier than Control animals. At 160 days, testis weight did not differ between groups but, due to the differences in body mass, relative testis weight, and thus gonadosomatic index, were smaller in the HFat group than in the Control group (Table 3).

Testis morphometry

In both groups, the testicular parenchyma showed a normal structure with seminiferous tubules and interstitial tissue, although some images show alterations in seminiferous tubules in the HFat group. Seminiferous tubule diameter was greater in the HFat group than in the Control group (Table 3, Fig. 2*a*, *b*). Compared to the Control group, the HFat group had greater numbers of Sertoli cells (Table 3, Fig. 2*c*, *d*) and Ki-67-positive spermatogonial cells (Table 3, Fig. 2*e*, *f*).

Table 3. Physiological and morphometric variables, and protein and gene expression for apoptosis and transcription factors, in the testis of adult rat pups born to mothers fed a Control (standard) diet or a high-fat diet (HFat) during pregnancy and lactation.

		Control	HFat	*P*-value	
Body mass at birth (g)		6.3 ± 0.7	5.5 ± 0.4	0.01	*
Body mass at puberty (g)		166.0 ± 4.3	144.0 ± 4.0	0.0078	**
Age at puberty (days)		37.0 ± 0.2	35.0 ± 0.2	0.008	**
Adult body mass at 160 days (g)		575.0 ± 10.0	771.0 ± 35.0	0.0001	***
Testis mass (g)		4.3 ± 0.2	4.5 ± 0.1	0.4	
Relative testis mass (g/g)		0.0075 ± 0.0003	0.0060 ± 0.0003	0.008	**
Gonadosomatic index (%)		0.75 ± 0.03	0.60 ± 0.03	0.008	**
Plasma androstenedione (ng/mL)		0.18 ± 0.02	0.20 ± 0.01	0.4	
Plasma testosterone (ng/mL)		1.47 ± 0.22	1.71 ± 0.38	0.5	
Testis morphometry					
Seminiferous tubule diameter (μm)		198.0 ± 6.3	211.0 ± 4.0	0.03	*
Sertoli cells (number per transverse section of seminiferous tubule)		9.5 ± 0.2	16.0 ± 0.3	0.001	***
Spermatogonia (Ki-67-positive cells per transverse section of seminiferous tubule)		12.8 ± 0.9	18.9 ± 1.1	0.0006	***
Testis protein immunoexpression (% area immunostained)					
Transcription factors	ETV5 (%)	3.5 ± 0.2	6.1 ± 0.4	0.0001	***
	GDNF (%)	14.3 ± 0.6	7.6 ± 0.6	0.0001	***
	IGF-1 (%)	4.9 ± 0.3	3.3 ± 0.3	0.01	*
Apoptosis proteins	Caspase-3 (%)	13.0 ± 0.5	15.6 ± 0.6	0.001	**
	Bax (%)	14.0 ± 0.6	14.2 ± 0.6	0.9	
	Bcl-2 (%)	21.8 ± 0.7	29.8 ± 0.8	0.0001	***
Testis gene expression (mRNA; ng/μL)					
Apoptosis factors	Caspase-3	0.50 ± 0.04	0.40 ± 0.09	0.4	
	Bax	0.38 ± 0.02	0.35 ± 0.01	0.26	
	Bcl-2	0.33 ± 0.03	0.19 ± 0.03	0.01	*

Data are expressed as mean ± s.e.m. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

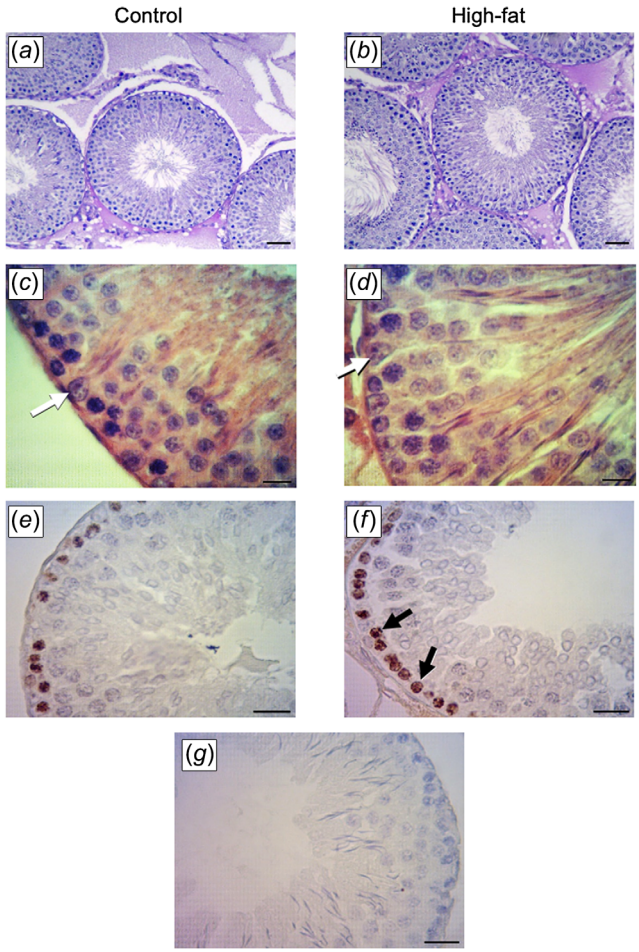

Fig. 2. Testis histology in male rats born to mothers fed either the Control or the High-fat diet during pregnancy and lactation. (*a*, *b*) Hematoxylin–eosin-stained seminiferous tubules (magnification, 100×; scale bar, 50 µm). (*c*, *d*) Sertoli cells (white arrows) in hematoxylin–eosin-stained seminiferous tubule cross-sections (magnification, 600×; scale bar, 20 µm). (*e*, *f*) Ki-67-positive spermatogonia (black arrows; magnification 400×; scale bar, 20 µm. (*g*) Negative control for immunohistochemistry.

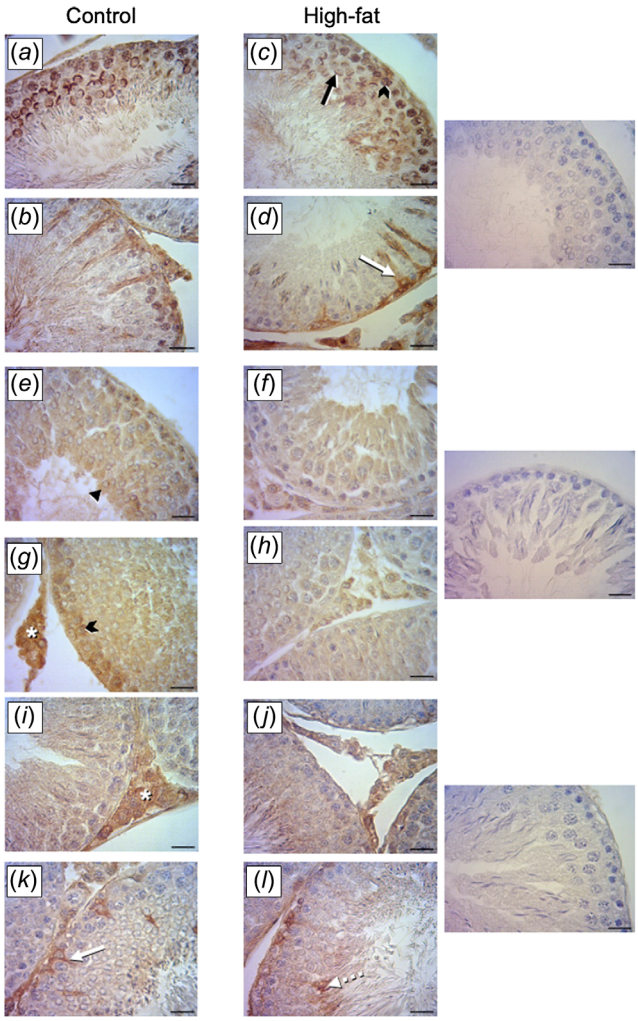

Fig. 3. Immunohistochemical localisation transcription factors in the testis in rats born to mothers fed either the Control or the High-fat diet during pregnancy and lactation. (*a–d*) Brown immunostaining for transcription variant 5 (ETV5) evident in the acrosome region in round spermatids (black arrow), spermatocytes (black arrowhead) and Sertoli cells (white arrows). (*e–h*) Brown immunostaining for glial-cell line-derived neurotrophic factor (GDNF) evident in elongated spermatids (black triangle), spermatocytes (black arrowhead) and Leydig cells (asterisk). (*i–l*) Brown immunostaining for insulin-like growth factor I (IGF-1) in Leydig cells (asterisk) and in Sertoli cells (intense immunostaining; white arrow) and spermatids (segmented arrow). Negative controls for immunohistochemistry are presented in the three panes on the right (magnification, 400×; scale bar, 20 µm).

Testis protein immunoexpression

Immunoexpression of the transcription factors, ETV5 and GDNF, was clearly evident in both groups. The ETV5 was localised in the spermatogonia, spermatocytes and the acrosome region of round spermatids, elongated spermatids and Sertoli cells (Fig. 3*a–d*). The IA for ETV5 was greater in the HFat group than in the Control group. For GDNF, immunoexpression was localised in cytoplasm of Sertoli cells, spermatogonia and Leydig cells (Fig. 3*e–h*). The IA for GDNF was lower in the HFat group than in the Control group.

The IGF-I immunoexpression was localised in the Leydig cells, the acrosome region of sperm and in Sertoli cells (Fig. 3*i–l*). The IA for IGF-I was lower in the HFat group than in the Control group.

Active caspase-3 immunoexpression was evident in round and elongated spermatids (Fig. 4*a–d*). The IA for caspase-3 was greater in the HFat group than in the Control group. For Bax, immunoexpression was localised in the cytoplasm of spermatogonia, elongated spermatids and Sertoli cells (Fig. 4*e–h*). The IA for Bax did not differ between dietary treatments. For Bcl-2, immunoexpression was detected in

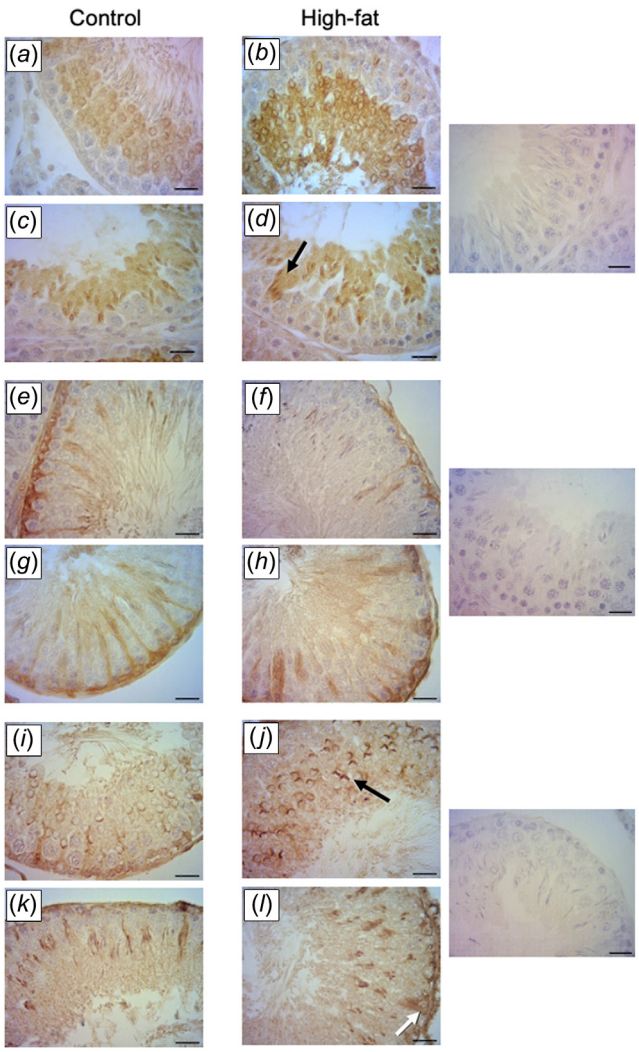

Fig. 4. Effects of maternal high-fat diet during pregnancy and lactation in apoptosis proteins in the testis. (*a–d*) Brown immunostaining for caspase-3 in spermatids (black arrows) and spermatozoa in the lumen. (*e–h*) Brown immunostaining for Bax evident in Sertoli cells and spermatogonia. (*i–l*) Brown immunostaining for Bcl-2 evident in acrosome region of round spermatids (black arrow) and in Sertoli cells (white arrow). Negative controls for immunohistochemistry are presented in the three panes on the right (magnification, 400×; scale bar, 20 μm).

round spermatids, elongated spermatids and Sertoli cells (Fig. 4*i–l*). The IA for Bcl-2 was greater in the HFat group than in the Control group (Table 3).

Testis gene expression

The testis levels of caspase-3 mRNA and Bax mRNA did not differ between dietary treatments. In contrast to Bcl-2 protein, the concentration of Bcl-2 mRNA was lower in the HFat group than in the Control group (Table 3).

Discussion

The maternal high-fat diet had significant consequences for the adult's male offspring including an earlier onset of puberty at a lighter body mass, followed by rebound growth leading to a heavier adult body mass. These outcomes were accompanied by major impacts on testis function, with significant alterations in testicular morphometry, including an increase in the diameter of seminiferous tubules that was caused, at least in part, by increases in the numbers of Sertoli cells and spermatogonial germ cells. In addition, apoptosis was increased in spermatocytes and spermatids. Thus, the maternal high-fat diet disrupted the balance of apoptosis and proliferation in the germ cells, challenging the ability of Sertoli cells to support germ cell development.

The body mass of the offspring was affected, with the HFat group being lighter at birth and at puberty, although the difference reversed as adulthood approached. These outcomes probably reflect general metabolic effects of the high-fat maternal diet on the offspring as seen in recent studies (Rodríguez-González *et al.* 2023). A generalised effect on metabolism could thus have contributed to effects in the developing testis.

Testis mass at adulthood (160 days) did not differ between groups but body mass was approximately 35% greater in the HFat group than in the Control group. Therefore, relative testis weight, and thus gonadosomatic index, was smaller in the HFat group than in the Control group. This outcome was accompanied by changes in morphometry, including an increase in the diameter of the seminiferous tubules, as reported for a previous study of rats fed high-fat diets during pregnancy (Campos-Silva *et al.* 2018). The increase in tubule diameter can be explained by increases in the number of Ki-67-positive spermatogonia (evidence of proliferation) and increases in the anti-apoptotic Bcl-2 protein. Germ cell proliferation would also be promoted in the high-fat group by an increase in the production by Sertoli cells of ETV5 that is essential for spermatogonial stem cell self-renewal (Morrow *et al.* 2007; Tyagi *et al.* 2009).

The production of IGF-I, a key growth factor involved in the regulation of spermatogenesis, is reduced by a maternal high-fat diet, leading to negative consequences for spermatocytes, early spermatids and spermatozoa, where IGF-I receptors have been previously reported for a variety of species (Vannelli *et al.* 1988; Griffeth *et al.* 2014). It is of interest to compare the outcomes of the present study with those from our recent work on the effects of maternal undernutrition during pregnancy or lactation. Notably, undernutrition evoked an opposite effect by increasing IGF-I production, whereas both dietary treatments evoked a decrease in GDNF (Pedrana *et al.* 2020, 2021).

With respect to apoptosis, Bcl-2 is known to play an anti-apoptotic role in the regulation of the balance between proliferation and programmed cell death. The maternal

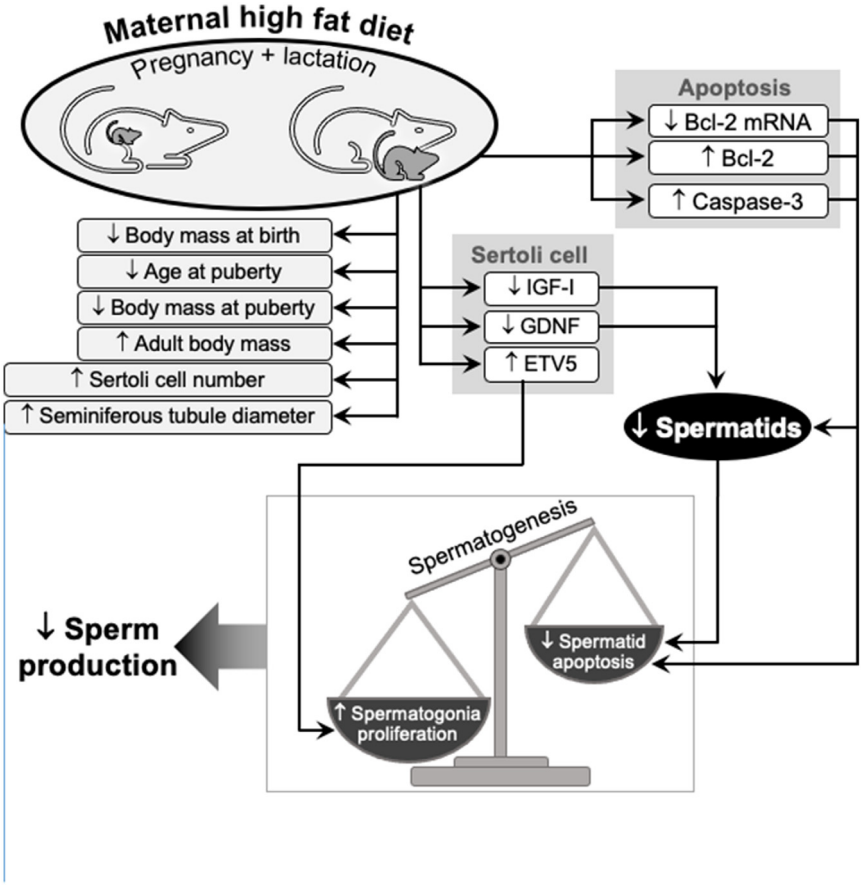

Fig. 5. Schematic summary of the effects of a maternal high-fat diet on the testis in adult offspring: morphometry and growth; factors that affect germ cell proliferation and apoptosis; and the consequences for Sertoli cell regulation of spermatogenesis. Essentially, a maternal high-fat during pregnancy and lactation leads to an imbalance between proliferation and apoptosis in germ cells, affecting the number of spermatogonia produced, with flow-on effects to spermiogenesis and fertility.

high-fat diet increased the amounts of Bcl-2 protein in Sertoli cells, spermatocytes and spermatids, but decreased the amount of Bcl-2 mRNA in testis of the adult offspring. This apparent contradiction can be explained by the well-established post-transcriptional modification of Bcl-2 protein (Greenbaum *et al.* 2003; Ramazi and Zahiri 2021). Indeed, we had previously shown that immunoexpression of Bcl-2 protein caspase-3 increase in parallel (Pedrana *et al.* 2021), consistent with Bcl-2 preventing apoptosis by blocking the release of cytochrome C from the mitochondria. In turn, pores induced by Bax permit the release of mitochondrial cytochrome C into the cytosol, subsequently activating the caspase cascade that leads to apoptosis (Singh *et al.* 2019).

Therefore, the increase in active caspase-3 protein in the spermatocytes and spermatids in the adult offspring in the high-fat diet treatment would be expected to reduce sperm output. Indeed, a similar dietary treatment was shown to reduce spermatid number and daily sperm production in offspring in early adulthood (Sertorio *et al.* 2022) and, recently, TUNEL was used to demonstrate an increased testicular apoptosis in 60 day old offspring of mothers fed a maternal 'cafeteria' diet (Meneghini *et al.* 2022).

We conclude that a maternal high-fat diet, fed during pregnancy and lactation, has major and persistent effects on a variety of factors that regulate spermatogenesis, and therefore the production of spermatozoa, in the adult offspring (Fig. 5). Changes in the production by Sertoli cells of growth factors and transcription factors lead to an acceleration of stem cell renewal and therefore excessive proliferation of germ cells. At the same time, changes in apoptotic proteins in spermatocytes and spermatids lead to a decrease in the rate of apoptosis. This disruption in the balance of proliferation and apoptosis increases the germ cell population and this challenging the capacity of Sertoli cells to provide support for germ cell development. Ultimately, there is a reduction in the production of spermatozoa in the adult pups. The 'fetal programming' hypothesis, previously applied to the effects of maternal undernutrition during prenatal and postnatal life, with consequences for the reproductive health of the next generation, is also clearly relevant to other types of maternal malnutrition.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest. Graeme Martin is the Editor-in-Chief of *Reproduction, Fertility and Development*, but was blinded from the peer-review process for this paper. There are no other conflicts of interest.

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Author affiliations

[A]Facultad de Veterinaria, Universidad de la República, Montevideo, Uruguay.

[B]UWA School of Agriculture and Environment, and UWA Institute of Agriculture, University of Western Australia, Perth, WA, Australia.

[C]Liggins Institute, University of Auckland, Auckland, New Zealand.

[D]Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, Canada.

[E]Department of Obstetrics and Gynecology, McMaster University, Hamilton, ON, Canada.

[F]Department of Pediatrics, McMaster University, Hamilton, ON, Canada.

[G]Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, ON, Canada.