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Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice

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BACKGROUND: Obesity and related conditions, notably subfertility, are increasingly prevalent. Paternal influences are known to influence offspring health outcome, but the impact of paternal obesity and subfertility on the reproductive health of subsequent generations has been overlooked.

METHODS: A high-fat diet (HFD) was used to induce obesity but not diabetes in male C57Bl6 mice, which were subsequently mated to normal-weight females. First-generation offspring were raised on a control diet and their gametes were investigated for signs of subfertility. Second-generation offspring were generated from both first generation sexes and their gametes were similarly assessed.

RESULTS: We demonstrate a HFD-induced paternal initiation of subfertility in both male and female offspring of two generations of mice. Furthermore, we have shown that diminished reproductive and gamete functions are transmitted through the first generation paternal line to both sexes of the second generation and via the first generation maternal line to second-generation males. Our previous findings that founder male obesity alters the epigenome of sperm, could provide a basis for the developmental programming of subfertility in subsequent generations.

CONCLUSIONS: This is the first observation of paternal transmission of diminished reproductive health to future generations and could have significant implications for the transgenerational amplification of subfertility observed worldwide in humans.

Key words: paternal obesity / fertilization / DNA damage / sperm quality / oocyte quality

Introduction

Obesity associates with other health pathologies, such as metabolic syndrome and subfertility, and its consequences are responsible for a large burden on the health system. As greater than 60% of western adults are either overweight or obese and because this fraction is increasing, it represents an ever increasing proportion of the overall disease burden (Begg *et al*., 2007; Nguyen and El-Serag, 2010). Maternal obesity is well established as a cause of subfertility in women [reviewed by Koning *et al*. (2010)]. Although the impact of male obesity upon fertility in both humans (Jensen *et al*., 2004; Magnusdottir *et al*., 2005; Kort *et al*., 2006; Hammoud *et al*., 2008; Chavarro *et al*., 2010; MacDonald *et al*., 2010; Bakos *et al*., 2011a; Tunc *et al*., 2011) and mice (Ghanayem *et al*., 2010; Bakos *et al*., 2011b) is emerging as a key cause of impaired fertility, the impact on the fertility of subsequent generations sired by an obese male remains unclear.

Rates of obesity within men of reproductive age has tripled since 1970 (Chiang *et al*., 2009; Tamer Erel and Senturk, 2009; Bakos *et al*., 2011a), and ~80% of male partners seeking assisted reproductive technology intervention are overweight or obese (Bakos *et al*., 2011a). Although a high paternal BMI has been associated with increased BMI in children (Danielzik *et al*., 2002; Li *et al*., 2009), this phenomenon is usually ascribed to inherited genetic predisposition or an 'obesogenic' environment being shared by both father and child.

Paternal obesity can reduce sperm concentration and motility, concomitant with an increase in reactive oxygen species (ROS) and sperm DNA damage (Jensen *et al*., 2004; Magnusdottir *et al*., 2005; Kort *et al*., 2006; Hammoud *et al*., 2008; Chavarro *et al*., 2010;

MacDonald et al., 2010; Bakos et al., 2011b; Tunc et al., 2011). The detrimental effects of paternal obesity upon human sperm are recapitulated in mouse models, indicating parallel outcomes in both mice and humans, and they implicate non-classical genetic mechanisms (e.g. epigenetic) as a potential mode of inheritance.

Paternal non-genetic transmission that impacts upon offspring phenotype has been described in a number of settings (Chong et al., 2007; Dunn and Bale, 2009; Ng et al., 2010; Youngson and Whitelaw, 2011). Maternal high-fat diet (HFD) resulted in heavier male offspring with diabetes, which in turn was transmitted to the second generation (Dunn and Bale, 2009); however, impacts upon offspring reproductive viability were not investigated. Recently, it was shown that a chronic HFD-induced paternal obesity with diabetes resulted in impaired glucose homeostasis, restricted to female offspring. This phenotype was suggested to manifest from impaired pancreatic function due to reduced β−cell number and perturbed β−cell morphology, which was associated with altered epigenetic marks within the pancreas (Ng et al., 2010)

Although the precise mechanisms that transmit paternal exposures to perturbations in offspring health remain unclear, molecular changes to the sperm are clearly implicated [reviewed by Krawetz (2005) and Youngson and Whitelaw (2011)]. We have previously established in mice that paternal obesity, in the absence of diabetes, alters sperm oxidative stress (Bakos et al., 2011b), levels of DNA damage (Bakos et al., 2011b) and changes to the sperm epigenome via increased acetylation (Palmer et al., 2011) and differential microRNA content (Ohlsson Teague et al., 2011) These sperm traits are concomitant with reduced sperm function (Bakos et al., 2011b) and resultant embryos were developmentally impaired and had poorer survival rates (Mitchell et al., 2011a). Sperm DNA damage induced by γ-radiation has been demonstrated to be a heritable trait in a mouse model (Adiga et al., 2010). Further to this sperm abnormalities and alterations to sperm DNA methylation have been observed, heritable through to the third generation via the male lineage as a result of maternal exposure to an endocrine disruptor, vinclozolin (Guerrero-Bosagna et al., 2010). But whether the elevated levels of DNA damage and ROS and other molecular constituents of sperm can program the reproductive viability of subsequent generations remains untested.

Therefore, using C57Bl6 mice, we determined the effect of periconception paternal exposure of founder males (F_0) to a HFD, which induces obesity but not diabetes, on the reproductive viability of two subsequent generations of offspring. The transgenerational impact on reproductive health was investigated in both the first generation (F_1) and second generation (F_2) offspring raised in a non-obesogenic environment [i.e. control diet (CD)]. Both male and female F_1 offspring demonstrated subfertility phenotypes that were transmitted to both sexes of the F_2 generation via F_1 males and to F_2 males via the F_1 maternal lineage. This is the first demonstration of HFD-induced paternal initiation of intergenerational transmission of impaired reproductive function in offspring.

Materials and Methods

Animals and diet

To examine the effect of paternal HFD-induced obesity distinct from diabetes on offspring's reproductive viability, we used a strain of mouse (C57Bl6) previously shown to develop only obesity, i.e. an 90−120% increase in adiposity concomitant with an increase in total body weight in the absence of altered glucose homeostasis, when exposed to a HFD for 10 weeks (Fullston et al., 2010; Bakos et al., 2011b; Mitchell et al., 2011a; Palmer et al., 2011). All mice were obtained and housed by the University of Adelaide Laboratory Animal Services, Adelaide, Australia. The animal ethics committee of the University of Adelaide approved all experiments, and the animals were handled in accordance with the Australian Code of Practices for the Care and Use of Animals for Scientific Purposes. All mice were maintained at 24°C on a 14 h light, 10 h dark illumination cycle.

Five-week-old C57Bl6 male mice were allocated into two groups: either receiving a CD ($n = 10$) containing only 6% fat, 19% protein and 64.7% carbohydrate (SF04-057; Specialty Feeds, Glen Forrest, Australia); or HFD ($n = 10$) containing 22% fat, 0.15% cholesterol, 19% protein and 49.5% carbohydrate (SF00-219; Specialty Feeds), intended to mimic a western style fast food diet. These diets were matched for other nutritional content and were provided *ad libitum* as was water. Males were housed individually and maintained on these diets for 10 weeks. All assessments of sperm function were performed blinded to the diet group.

Founder males were mated with normal-weight females to produce the F_1 generation.

F_1 males and females were subsequently mated with normal-weight females and males, respectively, to generate the F_2 generation of mice. All mice, other than half of the F_0 males, were maintained on CD feed.

Male reproductive measures

Blood hormone analysis

For testosterone measurements, male mice were anaesthetized and a blood sample was obtained by cardiac puncture, plasma was separated by centrifugation. Plasma testosterone was quantified at the ANZAC Research Institute (Sydney, Australia) with a stable-isotope dilution liquid chromatography coupled with tandem mass spectrometry and atmospheric pressure photoionization method using an API 5000 instrument (AB SCIEX, Foster City, USA), as described previously (Harwood and Handelsman, 2009). Sample preparation involved liquid−liquid extraction with 1 ml of hexane:ethyl acetate (3:2 ratio) containing deuterated internal standards.

Calibrants and quality control samples (three levels) were prepared in 4% bovine serum albumin. Accuracy was assessed by spiked recovery of serum pools, and imprecision was assessed using the quality control samples.

Sperm motility and morphology

Spermatozoa were obtained from the vas deferens, transferred into GIVF medium (Vitrolife AB, Gothenburg, Sweden) and incubated at 37°C for 5−10 min before and during (heated microscope stage) assessment. Sperm concentration was determined using an improved Neubauer haemocytometer and applying the appropriate dilution factor as recommended by the World Health Organization laboratory manual for the examination of human semen and sperm−cervical mucus interaction (WHO, 1999). Sperm motility was determined manually under ×40 magnification at 20−25°C (i.e. room temperature) and expressed as a percent of sperm in a given sample that were motile. Duplicate samples of 200 sperm were counted for motility assessment (Bakos et al., 2011b). Mice were deemed to have low motility if <30% of their sperm were progressively motile. Morphology was assessed using sperm samples fixed with methanol:acetone (3:1) and subsequently stained with haematoxylin and eosin. At least 200 individual sperm per animal were classified as normal, or having a tail defect (bent or twisted tails) or head defect (large, small or deformed heads).

Sperm intracellular ROS

Intracellular ROS levels were assessed in progressively motile spermatozoa, to ensure that analysis was conducted on live sperm, as this dye requires cleavage by intracellular esterase to detect ROS levels. The probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA; Sigma, Lenexa, USA), which fluoresces when it binds to intracellular ROS was utilized as previously described (Nasr-Esfahani *et al*., 1990; Lane *et al*., 2002; Bakos *et al*., 2011b). Motile sperm were incubated with the 5 μM DCFDA for 15 min at 37°C. Spermatozoa were then washed twice and examined using a fluorescent microscope with photometer attachment. Each sperm was imaged individually and a fluorescence reading obtained. The mean relative fluorescence was measured for a minimum of 30 motile sperm per sample and the mean was calculated for each animal. The results were expressed as mean fluorescence units or normalized so that the average fluoresence in the CD mice became equal to 100 (Nasr-Esfahani *et al*., 1990; Lane *et al*., 2002; Bakos *et al*., 2011b).

Assessment of capacitation and acrosome reaction

Capacitation and acrosome reaction were measured using *Arachis hypogaea* (peanut) agglutinin (PNA) conjugated to Alexa Fluor 488 (PNA; Molecular Probes, Eugene, USA) as described previously (Baker *et al*., 2004; Bakos *et al*., 2011b). A sperm sample was used to determine the percentage of capacitating and acrosome-reacted motile sperm at the time of insemination. Briefly, sperm samples were first washed twice with phosphate-buffered saline (PBS) at 1000 g for 1 min. Sperm samples were then incubated with Lectin PNA Alexa fluorophore at a final concentration of 100 μl ml^{-1} for 30 min at room temperature. Samples were then washed twice with PBS at 1000 g for 1 min and then incubated with propidium iodide (PI) (1 mg ml^{-1}) to identify sperm nuclei. Smears were then washed with PBS twice. Exactly 10 μl of spermatozoa was then smeared on polylysine-coated slides and examined using a fluorescent microscope. Two individual filters were utilized to capture the nuclear signal (PI; excitation: 540–565 nm, emission: 605–660 nm) and the lectin PNA signal (conjugate Alexa Fluor 488; excitation: 465–495 nm, emission: 515–555 nm). Sperm samples were categorized as capacitated, non-capacitated or acrosome reacted as described previously using this method (Baker *et al*., 2004). A minimum of 200 sperm were counted per animal.

Sperm DNA damage

Levels of DNA damage in sperm was assessed using terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) as per (Bakos *et al*, 2011b). Briefly, spermatozoa were air-dried and fixed with methanol:acetic acid (ratio 1:3) for 1 h at room temperature and then permeabilized with 0.5% Triton X-100 in 0.5% sodium citrate for 1 h, and incubated with Cell Death Detection Kit (Roche, Mannhein, Germany) for 1 h at 37°C as per the manufacturer's instructions. Smears were stained with 1 mg ml^{-1} PI to identify sperm nuclei. The percent sperm DNA damage was calculated as the number of TUNEL-positive sperm divided by the total number of sperm nuclei. At least 200 sperm were counted for each sample as described previously (Bakos *et al*., 2011b). A male was classified as having sperm DNA damage if one or more sperm was TUNEL positive.

Sperm binding and fertilization rates

To assess sperm binding to the zona pellucida and fertilization rates, mature cumulus-enclosed oocytes were collected from 4-week-old C57Bl6 × CBA hybrid females 12 h following ovulation induction with an intraperitoneal injection of pregnant mare's serum gonadotrophin (PMSG; Folligon; Intervet, Bendigo, Australia) and hCG (Pregnyl; Organon, Oss, The Netherlands) administered 48 h apart (Lane *et al*., 2003). Cumulus–oocyte complexes (COCs) ($n \geq 45$ oocytes per male) were placed in 80 μl drops of GIVF at 6% CO_2, 5% O_2 and 89% N_2 at 37°C. Sperm samples from males were collected from the vas deferens and capacitated for 1 h in GIVF medium (Vitrolife). After 1 h, oocytes were inseminated with 1×10^6 of motile sperm per ml and gametes co-incubated for 4 h. Sperm was removed from the drops containing the cumulus and oocytes at 4 h post-insemination. At 4 h post-insemination, sperm binding to oocytes was assessed by incubation for 3 min in bisbenzamide (25 μg ml^{-1}) followed by imaging under UV light. The number of sperm bound to the zona pellucida was assessed by counting sperm nuclei. At 6 h post-insemination, the number of fertilized oocytes was determined by the presence of two pronuclei within the oocyte following staining with bisbenzamide. Fertilization rates are expressed as the percentage of oocytes with two pronuclei from the total number inseminated.

Female reproductive measures

Cumulus expansion and oocyte meiotic maturation

Female offspring were synchronized for ovulation by injection with 10 IU PMSG (Intervet). Ovaries were collected 46 h post PMSG and COCs were aspirated from antral follicles in maturation medium (Lane *et al*., 2003) supplemented with 10% fetal calf serum (Invitrogen, Carslbard, USA), 50 IU FSH (Organon) and 10 μg ml^{-1} of epidermal growth factor (Sigma).

After 17 h of *in vitro* maturation cumulus expansion was assessed according to the 0–4 scale, and the cumulus expansion index was calculated (Fagbohun and Downs, 1990; Vanderhyden *et al*., 1990; Yeo *et al*., 2009). Using this scale, score 0 indicates no detectable response characterized by the detachment of cumulus cells from the oocyte to assume a flattened monolayer of fibroblastic appearance. A score of +1 indicates the minimum observable response where COCs remain spherical, cumulus cells have a glistering appearance, and most cells remain compacted around the oocyte. For score +2 complexes, only the outermost layers of cumulus cells have expanded; score +3 complexes have all layers except the corona radiata (cells most proximal to the oocyte) prominently expanded; and a score of +4 indicates the maximum degree of expansion including the corona radiata (Vanderhyden *et al*., 1990; Yeo *et al*., 2009).

Oocytes were denuded and then stained with 3 μM 4′-6-diamidino-2-phenylindole and nuclear status determined (Yeo *et al*., 2009).

ROS levels in metaphase II oocytes

The level of ROS in metaphase II (MII) oocytes was determined using a DCFDA (Sigma) fluorescence assay (Nasr-Esfahani *et al*., 1990; Lane *et al*., 2002). Oocytes were incubated for 30 min in 1 μM DCFDA, and the level of fluorescence measured using fluorescence spectroscopy. The relative fluorescence for each oocyte dihydrofluorescein was then expressed as a percent of control oocytes.

Measurement of ovulated oocytes mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was determined by staining MII oocytes with the mitochondrial stain, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide; Molecular Probes) at a concentration of 10 μM for 15 min at 37°C in the dark. Oocytes were then imaged using a confocal microscope. All images of fluorescently labelled oocytes were analysed using the Adobe Photoshop Pro software package (version 13; Adobe Systems, San Jose, USA). Mitochondrial properties were examined in the outer, intermediate and central regions of the oocyte as per Wakefield *et al* (2008). The mean red/green ratio pixel intensity was then determined within each of the three areas, which were then expressed as a percent of Inner from CD oocytes (Wakefield *et al*., 2008).

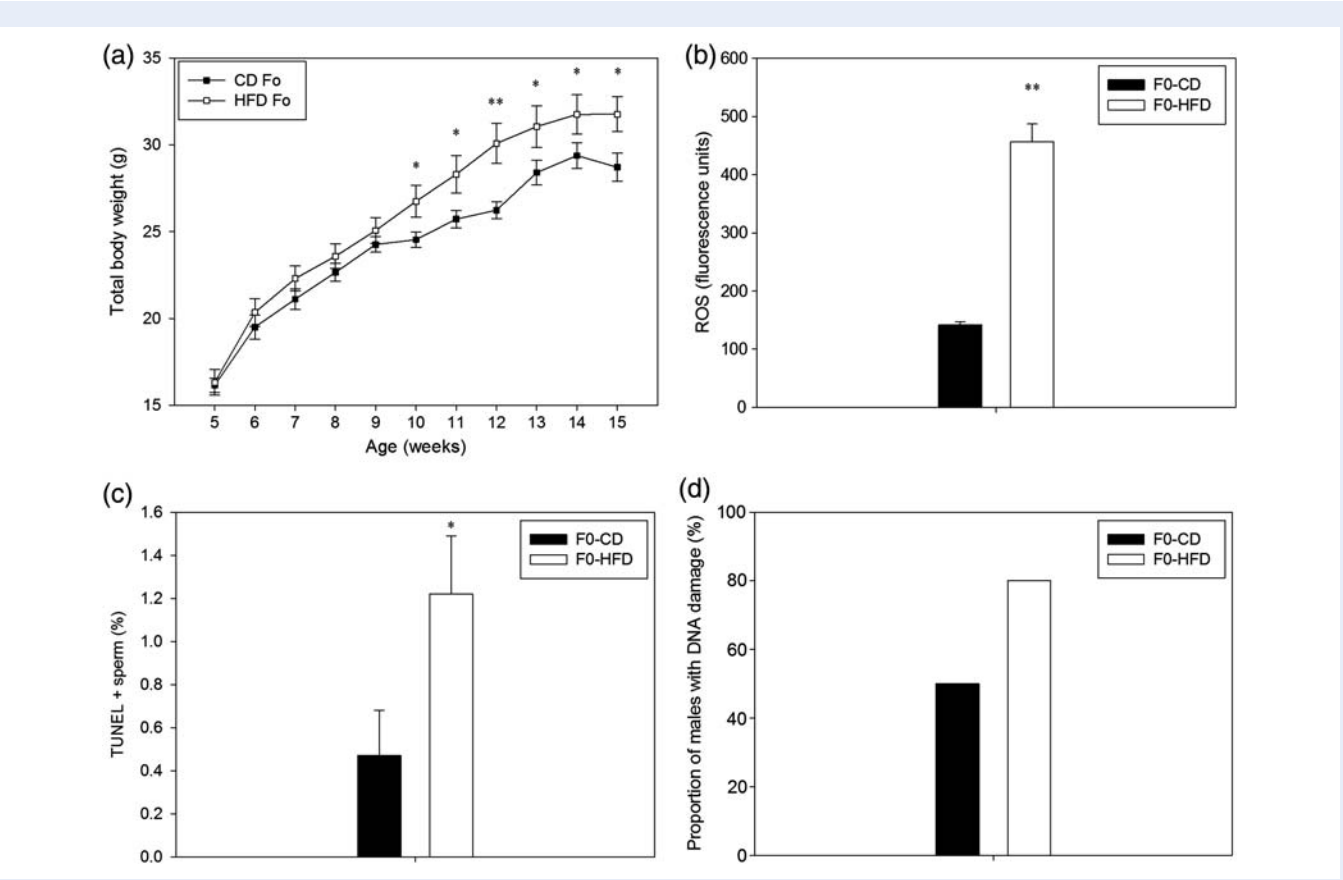

Figure 1 Total body weight and sperm parameters from F_0 males ($n = 10$). (**a**) Total body weight of F_0 males during the dietary treatments. Sperm from F_0 males fed either a CD or HFD were measured for (**b**) ROS, expressed as fluorescence units and sperm DNA damage presented as either (**c**) proportion of sperm with DNA damage or (**d**) proportion of animals with DNA damage. Data are presented as mean values ± SEM (*$P \leq 0.05$; **$P \leq 0.01$) and by repeated measures analysis $P = 0.003$ for body weight. Other founder male outcomes were analysed using Student's *t*-test or Mann–Whitney *U*-test.

Statistics

All data are presented as mean values ± SEM, where appropriate. For experiments examining founders, outcomes were analysed using Student's *t*-test or Mann–Whitney *U*-test for non-parametric data. Repeated measure analyses were performed where appropriate. For binomial data differences between groups were assessed by Fisher's exact test. For analysis of offspring data, analysis was performed by generalized linear modelling with parent identification included as a factor. Between treatment differences were assessed using least significant difference *post hoc* analysis. Levels of significance were set to $P \leq 0.05$.

Results

A HFD F_0 phenotype is confirmed

HFD F_0 males had increased total body weight, increased levels of ROS and an increased sperm DNA damage (% sperm TUNEL-positive CD F_0 0.47 ± 0.21%; HFD F_0 1.22 ± 0.27%; $P = 0.02$) (Fig. 1), although it must be noted that the majority of sperm did not give a positive signal for DNA damage by TUNEL. Concomitantly HFD F_0 displayed a significant increase in the percent of morphologically abnormal sperm (CD F_0 65.14 ± 1.97%; HFD F_0 81.42 ± 1.49%; $P = 0.0002$). These animals did not show any signs of altered glucose homeostasis by fasting blood glucose, glucose tolerance testing or plasma insulin levels (data not shown).

HFD fed males successfully mated with fewer females than CD males (CD 100%; HFD 60.5%; $P = 0.04$) therefore generating fewer successful pregnancies, although of the successful matings, there were no differences in time to mate (CD 2.90 ± 0.40 days; HFD 3.60 ± 0.40 days) gestational length (CD 21.40 ± 0.47 days; HFD 20.60 ± 0.26 days) or litter size (CD 8.50 ± 1.41; HFD 9.50 ± 2.15). Therefore, any consequences for offspring health are not a result of altered litter size and competition for nutrients *in utero* or altered gestation length and developmental maturity at birth.

A paternal founder HFD impairs reproductive health of F_1 offspring

F_1 males had no differences in testes and seminal vesicle weights or testosterone levels irrespective of founder male diet (Table I). Despite this male F_1 offspring fathered by HFD founder males had reduced sperm function including reduced motility, and increased sperm ROS and DNA damage (−18.1% $P = 0.04$,

Table I Summary of F_1 generation male's reproductive measures at 17 weeks of age.

Parameter measured	F_0 CD	F_0 HFD	*P*-value
Total body weight			
Post-mortem weight (g)	**35.1 ± 0.56**	**37.3 ± 0.68**	**0.02**
Reproductive organs	$n = 16$	$n = 16$	
Testis (g)	0.11 ± 0.002	0.12 ± 0.008	NS[a]
Epididymis (g)	0.04 ± 0.002	0.04 ± 0.002	NS
Seminal vesicles (g)	0.31 ± 0.008	0.29 ± 0.018	NS[b]
Sperm measures			
Progressive motility (%)	**76 ± 8.2**	**63 ± 7.5**	**0.04**
Animals with low motility (<30%) (%)[c]	6.7	33.3	0.07
Sperm with normal morphology (%)	72 ± 7.0	75 ± 3.7	NS
Reactive oxygen species (ROS) (%)[d]	**100 ± 1.1**	**118 ± 1.2**	**0.03**
Capacitation	472 ± 4.7	512 ± 8.42	NS
Acrosome reacted (%)	41 ± 4.0	32 ± 3.4	NS
Binding (# of sperm)	**59 ± 3.8**	**36 ± 4.1**	**0.0007**
DNA damage (% of sperm)	**0.4 ± 0.22**	**0.9 ± 0.28**	**0.04**
DNA damage (% of sperm)[e]	**100 ± 57.7**	**250 ± 75.1**	**0.04**
Males with sperm DNA damage (%)	**12.5**	**75.0**	**0.001**
Fertilization (%)	**42 ± 8.3**	**8 ± 5.8**	**0.009**
Testosterone (nmol l^{-1})	0.7 ± 0.18	0.8 ± 0.23	NS
In vivo mating			
Time to mate (days)	2.6 ± 0.46	2.4 ± 0.60	NS
Gestation length (days)	20.5 ± 0.33	20.2 ± 0.20	NS
Number of pups	11.6 ± 0.53	10.4 ± 0.93	NS

Data are presented as mean values ± SEM ($n = 16$).
Values that are significantly different appear on bold font.
[a]NS difference between CD/HFD is not significant ($P > 0.05$) for absolute weight or proportion of total body weight.
[b]Value is significantly different as a proportion of total body weight ($P < 0.05$).
[c]Proportion of animals with <30% progressively motile sperm.
[d]ROS levels are normalized to CD.
[e]DNA damage is normalized so CD = 100. F_1 offspring data presented here was analysed by the use of generalized linear modelling with parent identification included as a factor.

+18.0% $P = 0.03$ and +63.0% $P = 0.001$, respectively), compared with offspring sired by CD founder males (Table I). No differences were observed for mating behaviour, time to mate, gestational length or number of pups sired by F_1 males from CD and HFD F_0 males (Table I). These phenotypes mimicked the impairment that we have previously reported in their fathers with exposure to a HFD (Bakos *et al.*, 2011b) and confirmed on this cohort of founders (F_0; Fig. 1).

Similarly, reproductive measures of female F_1 offspring fathered by HFD fed founders were perturbed with reduced meiotic competence of oocytes (−24.6% $P = 0.04$) and altered mitochondrial MMP in all regions of the oocytes (+17.5% $P = 0.05$, +62.4% $P = 0.0005$ and +57.1% $P = 0.0006$; Table II), markers of subfertility that are consistent with females fed a HFD, despite being fed a CD. In contrast to ovary weights, total number of oocytes and cumulus cell expansion index did not differ irrespective of F_0 male diet. Further, no differences were observed for mating behaviour, time to mate, gestational length or number of pups sired by F_1 females from CD and HFD F_0 males (Table II).

A paternal founder HFD impairs reproductive health of F_2 offspring

F_2 offspring fathered by F_1 males

Grandsons

F_2 males had no differences in reproductive organ weights or testosterone levels irrespective of founder male diet. However, reproductive outcomes of F_2 males from a F_1 father sired by a HFD founder male demonstrated reduced sperm motility and increased levels of ROS (Table III), again mimicking their father's (Table I) and grandfather's phenotype (Fig. 1).

Granddaughters

F_2 females from a F_1 father sired by a HFD founder, had oocytes with increased oxidative stress and altered mitochondrial function when compared with F_2 females from CD fed founders (Table III). This

Table II Summary of F_1 generation female's reproductive measures at 17 weeks of age.

Parameter measured	F_0 CD	F_0 HFD	*P*-value
Total body weight			
Post-mortem weight (g)	**32.7 ± 0.83**	**36.2 ± 0.70**	**0.005**
Reproductive organ			
Ovary (right)[a] (g)	0.01 ± 0.001	0.01 ± 0.001	NS
Oocyte measures			
Oocytes collected after PMSG (#)	16.0 ± 3.02	16.2 ± 3.76	NS
GV to MII (%)	**89**	**64**	**0.04**
ROS (%)	100 ± 2.5	107 ± 4.0	NS
MMP (%)[b]-Outer	**199 ± 9.3**	**234 ± 13.4**	**0.05**
Middle	**121 ± 4.7**	**195 ± 8.3**	**0.0005**
Inner	**100 ± 2.9**	**156 ± 6.7**	**0.0006**
Cumulus cell expansion index	2.9 ± 0.09	3.0 ± 0.01	NS
In vivo mating			
Time to mate (days)	2.8 ± 0.49	1.8 ± 0.48	NS
Gestation length (days)	20.5 ± 0.29	20.3 ± 0.25	NS
Number of pups	11.8 ± 0.63	13.0 ± 1.00	NS

Data are presented as mean values ± SEM ($n = 16$); GV, germinal vesicle.
Values that are significantly different appear on bold font.
[a]Left ovary was used for reproductive measures.
[b]Mitochondrial membrane potential (MMP) as assessed by ratiometric analysis using JC1 stain, and normalized so mean CD value for inner region = 100. F_1 offspring data presented here was analysed by the use of generalized linear modelling with parent identification included as a factor.

was observed in the absence of differences in ovary weights, total number of oocytes collected and cumulus cell expansion index.

F_2 offspring born to F_1 females

Grandsons

Male F_2 offspring from F_1 females sired by a HFD founder male had smaller testes and seminal vesicles as a proportion of total bodyweight and reduced testosterone levels. These males also had perturbed sperm function with reduced motility, increased ROS with a greater percentage of animals exhibiting a severe motility defect (Table IV), the first demonstration of non-genetic transgenerational impairment of male fertility.

Granddaughters

Ovary weights, total number of oocytes and cumulus cell expansion index did not differ in female F_2 offspring born to F_1 females irrespective of F_0 grandfather diet. Further, there was no effect of founder male diet in terms of the meiotic progression of the oocytes; however, there was a reduction in ROS in oocytes from females originating from HFD founder males (Table IV), suggestive of reduced metabolic output.

Thus, a paternal founder HFD impairs reproductive health of F_2 predominantly male offspring from F_1 mothers, largely in parallel with and possibly attributable to the extent of their metabolic impairment (Fullston *et al.*, 2010). Founder HFD exposure through the maternal line, resulted in F_2 males who are obese, that also exhibited marked reproductive impairment similar to their HFD exposed grandfathers, in contrast to the F_2 female offspring, with no obvious metabolic or reproductive changes.

Discussion

We show for the first time that paternal exposure to a HFD in mice that causes paternal obesity, in the absence of diabetes, diminishes the reproductive health of both male and female F_1 offspring. Furthermore, the reproductive health of the F_2 generation was also compromised; with a severe male subfertility phenotype transmitted through the maternal F_1 lineage and compromised gametes of both F_2 sexes from the paternal F_1 lineage. This demonstrates that paternal obesity can contribute to intergenerational transmission of a decline in fertility, particularly in males.

A paternal exposure to a HFD that induced obesity without diabetes caused significant reproductive impairment, consistent with our previous findings in both mice (Bakos *et al.*, 2011b; Mitchell *et al.*, 2011a; Palmer *et al.*, 2011) and humans (Bakos *et al.* 2011a). Impaired HFD F_0 sperm had increased ROS and DNA damage, reduced motility, binding and fertilization concomitant with recent data demonstrating altered acetylation (Palmer *et al.*, 2011) and microRNA content (Ohlsson Teague *et al.*, 2011). Increased sperm ROS and DNA damage with reduced motility, binding and fertilization was transmitted to F_1 males. F_2 males from both F_1 male and female lineages had sperm with elevated ROS and reduced motility. Whether the reduction in motility observed in $F_0/F_1/F_2$ sperm is in part due to reduced viability remains to be determined. While only F_2 females from F_1 male linage were impaired with reduced mitochondrial function and elevated ROS, likely due to the impaired sperm parameters of the F_1 males that mimic the F_0 males, but in the absence of obesity.

A significant and novel finding of this study is the intergenerational transmission of impaired reproductive function through both parental

Table III Summary of F_2 generation sired by F_1 male's reproductive measures at 17 weeks of age.

Parameter measured	F_0 CD	F_0 HFD	*P*-value
F_2 males			
Total body weight (post mortem) (g)	33.2 ± 0.53	33.8 ± 1.84	NS
Testis (g)	0.10 ± 0.002	0.10 ± 0.003	NS
Epididymis (g)	0.04 ± 0.001	0.04 ± 0.002	NS
Seminal vesicles (g)	0.28 ± 0.008	0.25 ± 0.008	NS
Progressive sperm motility (%)	**94 ± 2.3**	**63 ± 10.4**	**0.045**
Animals with low motility (<30%) (%)	**0**	**50**	**0.01**
Sperm with normal morphology (%)	83 ± 2.8	84 ± 2.7	NS
ROS (%)	**100 ± 2.3**	**107 ± 1.8**	**0.006**
Sperm capacitation (%)	63 ± 3.9	72 ± 9.2	NS
Acrosome reacted (%)	23 ± 2.3	23 ± 8.7	NS
Sperm binding (#)	42 ± 2.4	45 ± 2.7	NS
DNA damage (% of sperm)	0.9 ± 0.30	1.1 ± 0.20	NS
DNA damage (% of sperm)[a]	100 ± 33.1	120 ± 21.7	NS
Males with sperm DNA damage (%)	75	100	NS
Testosterone (nmol l^{-1})	0.9 ± 0.26	0.9 ± 0.47	NS
F_2 females			
Total body weight (post mortem) (g)	29.8 ± 0.97	29.4 ± 0.62	NS
Oocytes collected after PMSG (#)	20.6 ± 1.75	22.6 ± 1.73	NS
GV to MII (%)	80.8 ± 3.61	73.4 ± 3.71	0.11
Oocyte reactive oxygen species (ROS) (%)	**100 ± 5.2**	**116 ± 7.1**	**0.03**
Oocyte MMP (%)–Outer	225 ± 11.9	235 ± 13.2	NS
Middle	**120 ± 6.7**	**165 ± 11.1**	**0.001**
Inner	**100 ± 5.7**	**120 ± 7.1**	**0.03**
Cumulus cell expansion index	2.7 ± 0.09	2.6 ± 0.10	NS

Data are presented as mean values ± SEM ($n = 8$).
Values that are significantly different appear on bold font.
[a]DNA damage is normalized so CD = 100. F_2 offspring data presented here was analysed by the use of generalized linear modelling with parent identification included as a factor.

lines sired by an obese father. Both males and females F_1 offspring of a HFD fed founder male had impaired reproductive function. In the case of the F_1 female offspring, changes observed in oocytes of reduced meiotic competence and mitochondrial function are reminiscent of perturbations reported for HFD-induced obesity in rodent females, such as reduced metabolic capacity (Igosheva *et al.*, 2010; Wu *et al.*, 2010). Despite being raised and maintained on CD F_1 females had increased adiposity, akin to obesity. Therefore, the effect of a paternal HFD on their F_1 female oocytes cannot be separated from the increased adiposity in these offspring, rather than a direct programming effect on the F_1 ovary. Regardless, the net effect is sub-optimal gametes that produced the F_2 generation. To this end, F_0 exposure to HFD through the maternal line resulted in F_2 male offspring who were obese, had smaller reproductive organs and reduced peripheral testosterone. This phenotype culminated in sperm defects of increased ROS and reduced motility, primarily a reduction in progressive motility, paralleling the reproductive impairment of their HFD-exposed grandfathers. In contrast, F_2 female offspring born to F_1 females had no obvious reproductive impairment. Based on the impaired reproductive function of the F_1 and F_2 generations derived from gametes with similar defects, we speculate that the reproductive health of the third generation (F_3) may also be compromised. Conversely, oocytes from F_2 females born to the female F_1 lineage appear relatively normal, with respect to germinal vesicle (GV) to MII progression, MMP and reduced levels of ROS. This highlights sex-specific outcomes in the F_2 generation born to the female F_1 lineage and as such sex-specific responses in transgenerational studies surrounding paternal peri-conception exposures need to be considered in future studies.

The transmission of increased sperm DNA damage caused by HFD-induced obesity in F_0 males to the male offspring in the next two generations (F_1 and F_2) is consistent with previous studies where DNA damage in sperm from founder male mice, induced by γ-irradiation, was inherited by their male offspring (Adiga *et al.*, 2010). Although the sperm perturbations observed here result from a paternal exposure to a HFD, we observed a similar phenomenon with the transmission of not only increased DNA damage but also increased ROS and decreased motility. DNA damage, as determined by TUNEL-positive sperm, was only present in a small proportion of $F_0/F_1/F_2$ sperm (i.e. 0.94–1.22%). This suggests that stochastically a sperm with DNA fragmentation was unlikely to fertilize an oocyte, but TUNEL is known to underestimate DNA

Table IV Summary of F_2 generation born to F_1 female's reproductive measures at 17 weeks of age.

Parameter measured	F_0 CD	F_0 HFD	*P*-value
F_2 males			
Total body weight (post mortem) (g)	**32.8 ± 0.83**	**36.2 ± 0.70**	**0.005**
Testis (g)	0.12 ± 0.004	0.11 ± 0.003	0.07[a]
Epididymis (g)	0.04 ± 0.004	0.04 ± 0.008	NS
Seminal vesicles (g)	0.31 ± 0.009	0.27 ± 0.015	NS[a]
Progressive sperm motility (%)	64 ± 4.8	51 ± 4.4	0.09
Animals with low motility (<30%) (%)	**0**	**38**	**0.03**
Sperm with normal morphology (%)	68 ± 4.7	78 ± 2.8	0.09
Sperm (ROS) (%?)	**100 ± 3.9**	**121 ± 4.9**	**0.003[b]**
Sperm capacitation (%)	68 ± 6.4	72 ± 93	NS
Sperm binding (#)	65 ± 6.5	52 ± 8.9	0.10[b,c]
Acrosome reacted (%)	23 ± 5.7	12 ± 2.6	0.08
DNA damage (% of sperm)	0.4 ± 0.14	1.0 ± 0.72	NS
DNA damage (% of sperm)[d]	100 ± 33.8	249 ± 172.2	NS
Males with sperm DNA damage (%)	63	63	NS
Testosterone (nmol l^{-1})	**0.9 ± 0.05**	**0.6 ± 0.09**	**0.05**
F_2 Females			
Total body weight (post mortem) (g)	27.9 ± 0.70	29.0 ± 1.11	NS
Oocytes collected after PMSG (#)	17.3 ± 2.40	16.1 ± 2.95	NS
GV to MII (%)	70 ± 4.6	66 ± 4.9	NS
Oocyte reactive oxygen species (ROS) (%)	**100 ± 5.7**	**77 ± 2.7**	**0.0005**
Oocyte MMP (%)–Outer	180 ± 12.4	231 ± 13.4	0.09
Middle	147 ± 8.6	136 ± 11.1	NS
Inner	100 ± 4.4	93 ± 5.3	NS
Cumulus cell expansion index	2.5 ± 0.12	2.3 ± 0.08	0.08

Data are presented as mean values ± SEM ($n = 8$).
Values that are significantly different appear on bold font.
[a]Difference is significant as a proportion of total body weight ($P < 0.05$).
[b]A negative correlation between ROS and sperm binding exists ($P = 0.03$).
[c]Mother effect observed, i.e. male F_2 offspring of some F_1 females were more affected.
[d]DNA damage is normalized so CD = 100. F_2 offspring data presented here was analysed by the use of generalized linear modelling with parent identification included as a factor.

damage in sperm (Mitchell *et al.*, 2011b). Therefore, this 0.94–1.22% of sperm displaying DNA damage by TUNEL may actually represent just the 'tip of the iceberg' with respect to the total DNA damage that might be detected with more sensitive or alternative techniques. This suggests that the sperm perturbations, even in the absence of male obesity (i.e. F_1 males), is sufficient to propagate this transmission through two subsequent generations. Further, obesity is not necessarily a prerequisite for the transmission of compromised gametes from F_1 males to F_2 females as our F_1 males were not obese. Indicating that F_1 sperm is programmed to be compromised by paternal obesity (F_0) and defects within it are also sufficient to propagate subfertility to the F_2 generation. This increased DNA damage and ROS may, in part, explain the subfertility in these obese F_0 males (Bakos *et al.*, 2011b; Palmer *et al.*, 2011) and the compromised development observed for embryos subsequently sired by them (Mitchell *et al.*, 2011a). We provide the first evidence that paternal obesity diminishes the reproductive viability of two generations of offspring in mice. Whether this data highlight a potential contributing mechanism that underpins the unexplained worldwide decline in human male fertility over the last 20 years remains to be determined.

It is likely that the observed transgenerational impairment of reproductive health resulted from the impaired metabolic health of the F_0 males (Fullston *et al.*, 2010), induces changes in epigenetic marks in the sperm of F_0 males. Given the impact on reproduction was transmitted through two generations, these alterations likely take the form of inherited persistent epigenetic marks through the germline that impact on gonad and gamete development and function (Skinner *et al.*, 2010). Indeed, the transmission of subfertility to the most reproductively compromised animals, i.e. F_0 male to F_1 female to F_2 male, not only tracks with obesity but is also suggestive of an X-linked mode of inheritance consistent with previous studies (Dunn and Bale, 2009). Preliminary studies have identified X-linked microRNAs that may exhibit differential content in sperm between HFD and CD F_0 males, which are predicted to target molecular pathways involved in gameteogenesis and embryo development (Ohlsson Teague *et al.*,

2011). In addition other epigenetic marks such as methylation, the global reduction of which has been previously associated with infertility (Tunc and Tremellen, 2009), might also play a role in the reduction of fertility seen in the F_0 males. Further investigation and thorough characterization of the epigenetic alterations to germ cells that result from a paternal HFD are warranted.

This is the first demonstration of HFD-induced paternal initiation and intergenerational transmission of diminished reproductive function with further transmission through both F_1 parental lines to F_2 generations. It can only be speculated that the reproductive viability of offspring sired by obese males raised in a similar obesogenic environment as that of their fathers, may well be further diminished. We have demonstrated a clear negative impact on the reproductive health of F_1 offspring sired by obese males, which persists into the subsequent F_2 generation, representing a potential pathway for amplification of the worldwide decline in fertility.

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Authors' roles

T.F., N.O.P., J.A.O., M.M. and M.L. designed the experiments. T.F., N.O.P., M.M., H.W.B. and M.L. executed the experiments. All authors contributed to data analysis. T.F. and M.L. prepared the manuscript. All authors reviewed and approved the final manuscript.

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Conflict of interest

None declared.

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