ORIGINAL ARTICLE

Male Reproduction

Metformin treatment of high-fat diet-fed obese male mice restores sperm function and fetal growth, without requiring weight loss

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Male obesity is associated with subfertility and increased disease risk of offspring. It is unknown if effects can be reversed through pharmacological interventions. Five- to 6-week-old C57BL6 male mice were fed control diet ($n = 10$, CD) or high-fat diet ($n = 20$, HFD) for 16 weeks. Animals fed with a HFD were then allocated to continuation of HFD ($n = 8$) or HFD with metformin 28 mg kg^{-1} day^{-1} ($n = 8$) for 6 weeks. Animals fed with CD continued on a CD ($n = 9$). Males were mated with fertile C57BL6 females for the assessment of pregnancy and fetal growth. Sperm motility, spermatozoa and testicular morphology, sperm-zona pellucida binding, sperm reactive oxygen species (ROS) (intracellular [DCFDA], superoxide [MSR], and oxidative DNA lesions [8OHdG]), and mitochondrial membrane potential (JC1) were assessed. Metformin treatment of HFD males improved glucose tolerance (+12%, $P < 0.05$) and reduced Homeostatic Model Assessment of Insulin Resistance (HOMA-IR; −36%, $P < 0.05$). This occurred in the absence of a change in body weight or adiposity. Metformin treatment of HFD-fed males restored testicular morphology (+33%, $P < 0.05$), sperm motility (+66%, $P < 0.05$), sperm–zona pellucida binding (+25%, $P < 0.05$), sperm intracellular ROS concentrations (−32%, $P < 0.05$), and oxidative DNA lesions (−45%, $P < 0.05$) to the levels of the CD males. Metformin treatment of HFD fathers increased fetal weights and lengths compared with those born to HFD fathers (+8%, $P < 0.05$), with fetal lengths restored to those of fetuses of CD males. Short-term metformin treatment in men who are obese could be a potential intervention for the treatment of subfertility, without the need for a reduction in body weight/adiposity.

INTRODUCTION

The incidence of obesity in men of reproductive age is increasing, with 28% of expectant fathers in Australia[1] and 33% of expectant fathers in the USA[2] classified as obese. It is now widely accepted that male obesity is associated with subfertility, increasing time to conception, and the need for assisted reproductive technologies to achieve a pregnancy.[3] This is likely due to the negative effects of obesity on semen quality, reducing sperm counts, motility, oocyte binding, increasing sperm reactive oxygen species, and DNA damage as well as altering the sperm microenvironment of seminal plasma.[4,5] Not only do these factors affect pregnancy, but they also influence the health of the next generation, altering birth weights in both animal models[6–10] and humans,[11,12] increasing offspring susceptibility to metabolic syndrome,[6,7,13] subfertility,[14] fatty liver,[15] kidney disease,[16] and hypertension,[17] while decreasing offspring cognitive function.[18] Therefore, understanding how to reverse these impacts on spermatozoa is vital, to break this chronic disease transgenerational cycle.

We have previously shown that diet or exercise interventions in our mouse model of obesity (with high-fat diet-feeding mimicking a Western fast-food diet), reduce adiposity, improve metabolic profile and sperm function, and reverse the adverse early embryo/fetal development and offspring outcomes.[8,19–21] Similar, positive findings have been shown in human studies of diet/exercise interventions in men who are obese.[22,23] However, one limitation of lifestyle interventions is long-term compliance and remission. Little information is known about whether pharmacological interventions can illicit similar positive changes to fertility and sperm function in men who are obese.

Our animal models of obesity have shown that systemic glucose control without overt metabolic syndrome influences sperm function and fertility irrespective of adiposity[8,19–21] and thus may provide a target for pharmacological intervention for the effects of obesity on spermatozoa. Metformin is a commonly used glucose-lowering drug for the treatment of Type II diabetes. Its main mode of action is to inhibit liver gluconeogenesis and increase insulin-mediated glucose uptake in skeletal muscle.[24] Treatment with metformin of streptozotocin-induced diabetic male rats has been previously shown to improve sperm quality (increased motility and reduced DNA damage) and increase testicular function (increased Leydig cell counts and steroidogenesis-related enzymes).[25] Further, metformin treatment can reduce oxidative stress and apoptotic markers (malondialdehyde and caspase-3, catalase, superoxide dismutase, and glutathione peroxidase) in testicular tissue after testicular torsion of male rats.[26] In humans, metformin treatment

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increases normal sperm morphology and chromatin packaging in men with hyperinsulinemia.[27] Therefore, the aim of our study was to determine if treatment of high-fat diet-fed obese male mice with metformin could improve sperm function.

MATERIALS AND METHODS

Animals, diet, and metformin treatment
Five-week-old male C57BL6 mice ($n = 30$) were randomly assigned to one of the two diets for an initial period of 16 weeks: (1) control diet (CD), low in saturated fat (0%, diet comprises 21% monosaturated fat from canola oil [6 g per 100 g]; SF16-137; Specialty Feeds, Perth, Australia) ($n = 10$), or (2) high-fat diet (HFD), high in saturated fat (40% saturated fat from clarified butter [21 g per 100 g] and nutrient matched; SF16-136; Specialty Feeds) ($n = 20$). Diets used in the study have been previously shown to increase adiposity.[28] After the initial feeding period, males allocated to the HFD were again allocated for further 6 weeks to either HFD with standard drinking water (HFD, $n = 8$) or HFD supplemented with 0.15 mg ml^{-1} of metformin (1,1-dimethylbiguanide hydrochloride, D150959, Sigma-Aldrich, St. Louis, MO, USA) in their drinking water (HFD + metformin, $n = 8$). On the basis of the average water consumption of 6 ml water per day per C57BL6 male,[29] each male was calculated to receive a mean metformin dose of 0.9 (standard deviation [s.d.]: 0.1) mg, which approximates a standard adult human dose of 2000 mg day^{-1} (28 mg kg^{-1} day^{-1}). This intervention method was based on a previous study assessing metformin therapy in the treatment of polycystic ovarian syndrome on oocyte quality in an obese female mouse model.[30] Mice allocated to the CD during the initial feeding period were also fed a CD during the interventional (6 weeks) period to be used as normal-weight controls (CD, $n = 9$). The 6-week interventional period was chosen as it approximates one full round of spermatogenesis in the mouse (approximately 35 days).[31–33]

Animal ethics approval
The use and care of all animals in the study was approved by the Animal Ethics Committee of The University of Adelaide (Adelaide, Australia) under the guidance of the Australian code for the care and use of animals for scientific purposes (8th edition, 2013).

Glucose tolerance testing (GTT) and insulin tolerance testing (ITT)
GTT was performed after a 6-h fast at 15 and 21 weeks by intraperitoneal (IP) injection of 2 g per kg body weight of 25% (w/v) D-glucose solution. ITT was performed at 16 and 22 weeks during a fed state by IP injection of 1.0 IU of human insulin (Actapid®, Novo Nordisk, Bagsvaerd, Denmark). Tail blood glucose concentrations were measured in a glucometer (Hemocue, Angelholm, Sweden) at time points 0 (prebolus basal), 15, 30, 60, and 120 min. Data were expressed as mean blood glucose concentration per group as area under curve (AUC) for GTT and area above the curve (AAC) for ITT. GTTs and ITTs were followed as per Fullston et al.[7]

Body composition
Individual body weights were recorded weekly during both initial and postinterventional periods. After 22 weeks of feeding, renal fat, dorsal fat, omental fat, gonadal fat, testes, seminal vesicles, liver, kidneys, spleen, and pancreas were collected and weighed postmortem.

Metabolites and hormone analysis
After 22 weeks on the diet, males were fasted overnight, and blood plasma (approximately 500 µl) was collected postmortem by a cardiac puncture under anesthesia with 5% (w/v) Avertin (2-2-2

tribromethanol, Sigma Aldrich). Individual animal plasma cholesterol (total cholesterol, high-density lipoprotein [HDL], low-density lipoprotein [LDL], and triglycerides) and glucose were measured on a Cobas Integra 400 plus automated sampler system (Roche, Basel, Switzerland) as per the manufacturer's instructions. Individual animal insulin, c-peptide, and leptin were assayed with the Merck Milliplex MAP Mouse Metabolic Panel assay (Merck, Kenilworth, NJ, USA). Individual animal C-reactive protein (CRP) was measured using the commercially available Mouse CRP Quantikine enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems, North East, MN, USA), while testosterone was assayed with the commercially available testosterone rat/mouse ELISA (Demeditec Diagnostics, Kiel, Germany). The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was determined from the following formula: plasma insulin (mmol l^{-1}) × blood glucose (mmol l^{-1})/22.5.[34]

Collection of mouse spermatozoa
Spermatozoa were collected postmortem from the right cauda epididymidis and ductus deferens and expressed into 0.5-ml G-IVF medium (Vitrolife, Gothenberg, Sweden) and incubated for at least 10 min in 6% (v/v) CO_2 and 5% (v/v) O_2 at 37°C.[35]

Sperm motility and morphology
Sperm motility and morphology were assessed in accordance with the WHO guidelines[36] (at least 200 spermatozoa counted for each sample). Sperm motility was assessed in a light microscope classifying 200 spermatozoa per animal as progressively motile, nonprogressively motile, or immotile. Sperm morphology was assessed in a blind fashion on samples fixed with a 3:1 ratio of methanol to acetone and stained with hematoxylin and eosin (Sigma-Aldrich). For identifying sperm morphology, individual spermatozoa were classified as normal or with a tail defect (bent tails and twisted tails) or head defect (large heads, small heads, and deformed heads) as per Palmer et al.[19] Morphology was expressed as percentage of normal forms.

Sperm–zona pellucida binding
Sperm binding to the zona pellucida was performed as described by Bakos et al.[35] Briefly, mature cumulus-enclosed oocytes (COCs) were collected from 4-week-old Swiss female mice 12 h following superovulation with intraperitoneal injections of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) administered 48 h apart. The spermatozoa ($n = 9$ CD, $n = 8$ HFD, and $n = 8$ HFD + metformin; 30×10^4 ml^{-1}) samples were co-incubated with ≥10 COCs in G-IVF for 1 h at 37°C, 6% CO_2, and 5% O_2. At 1 h postinsemination, the oocytes were gently washed and moved into a new medium drop to remove unbound spermatozoa, and sperm binding was determined by counting the number of spermatozoa bound to the oocyte by phase-contrast microscopy (Olympus BX50, Shinjuku, Tokyo, Japan). At least ten oocytes were analyzed per sperm sample.

Sperm intracellular ROS assay (DCFDA)
Intracellular reactive oxygen species (ROS) levels were assessed on progressively motile spermatozoa as per Bakos et al.[37] Briefly, motile spermatozoa were incubated with 5 µmol l^{-1} DCFDA (2',7'-dichlorodihydrofluorescein diacetate; Sigma, Lenexa, KA, USA) for 15 min at 37°C, washed twice in G-IVF minus albumin (Vitrolife), and examined using a photometer attachment (Leitz MPV-Combi) on a fluorescence microscope (Leica BMIRB, Wetzlar, Germany) to derive a fluorescence reading for individually imaged spermatozoa. A minimum of twenty motile spermatozoa were measured per animal and expressed as relative fluorescent units.

Sperm superoxide assay (MitoSOX Red)

The intracellular generation of superoxide ROS was determined by using MitoSox Red (MSR; Molecular Probes, Eugene, OR, USA) as per Koppers et al.[38] The spermatozoa (10^6 ml^{-1}) were incubated with 0.05 μmol l^{-1} MSR and 2 μmol l^{-1} SytoxGreen (vitality stain) for 30 min at 37°C, 6% (v/v) CO_2, and 5% (v/v) O_2. A negative control where the spermatozoa were incubated only in SytoxGreen was included. MSR and SytoxGreen fluorescence were measured on a FACSCanto flow cytometer (BD Bioscience, North Ryde, Australia). Nonspecific sperm events were gated out, and 20 000 cells were examined per sample. The results were expressed as percentage of live spermatozoa positive for MSR.

Sperm oxidative DNA damage assay (8OHdG)

Oxidative DNA damage was assessed in the spermatozoa as per McPherson et al.[39] Briefly, the spermatozoa were fixed to polylysine slides with a 3:1 ratio of methanol to acetone for 5 min and then permeabilized for 45 min in 0.5% (v/v) Triton-X-100 (Sigma-Aldrich). The slides were then washed in PBS, and sperm nuclei were decondensed for 60 min at 37°C in 1 mol l^{-1} HCI and 10 $mmol$ l^{-1} Tris buffer containing 5 $mmol$ l^{-1} dithiothreitol. Sperm nuclei were then denatured in 6 mol l^{-1} HCI/0.1% Triton-X-100 for 45 min at room temperature followed by neutralization with 100 μmol l^{-1} Tris HCI. The sperm slides were then incubated in 1:100 (0.5 μg) mouse anti-8 hydroxyguanosine (8OHdG) antibody [N45.1] (ab48508, Abcam, Cambridge, UK) in 10% (v/v) donkey plasma (Sigma-Aldrich) in PBS overnight at 4°C followed by washes with PBS and incubation in 1:100 Biotin-SP-conjugated AffiniPure F(ab')$_2$ fragmented donkey anti-mouse IgG (H + L) (715-065-150, Jackson ImmunoResearch, Baltimore, PA, USA) at room temperature for 2 h. Following PBS washes, the sperm slides were incubated in 1:100 (10 μg) Cy3-conjugated streptavidin (016-160-084, Jackson ImmunoResearch) for 1.5 h at room temperature followed by nuclear counterstaining with Hoechst (Sigma-Aldrich). The spermatozoa were loaded in glycerol/prolong gold solution (Invitrogen, Carlsbad, CA, USA) and viewed by epifluorescence microscopy. A negative control was added where the primary antibodies were omitted from the reaction. For 8OHdG staining, 100 spermatozoa per animal were assessed as either positive or negative for staining, and this was expressed as a percentage of positive spermatozoa.

Sperm mitochondrial membrane potential assay (MMP) (JC-1)

Sperm mitochondrial membrane potential was determined by using the ratiometric dye JC-1 (Molecular Probes) as previously described by Koppers et al.[38] The spermatozoa (1×10^6 ml^{-1}) were incubated with 2 μmol l^{-1} JC-1 for 30 min at 37°C, 6% (v/v) CO_2, and 5% (v/v) O_2. The samples were then washed and counterstained with 0.25 mg ml^{-1} propidium iodide (PI; to indicate dead cells to be excluded from the analysis). A negative control was also included, in which the spermatozoa were incubated in 10 μmol l^{-1} carbonyl cyanide 3-chlorophenylhydrazone to dissipate membrane potential before incubation in JC-1. JC-1 and PI fluorescence were measured on a FACSCanto flow cytometer (BD Bioscience). Nonspecific sperm events were gated out, and 20 000 cells were examined per sample. The results were expressed as the ratio of red-to-green fluorescence in live spermatozoa. A lower ratio of red to green is associated with increased mitochondria depolarization.

Testicular histology

The left testis was fixed in 4% (w/v) paraformaldehyde overnight and then placed into 70% (v/v) ethanol for storage. For sectioning, each testis was embedded in wax by standard methods, and 5-μm sections were cut and heat-fixed onto superfrost slides. Each slide contained four different 5 μm sections of testis that had been cut 50 μm apart to represent most of the testicular tissue. The sections were de-waxed in xylene twice for 5 min and rehydrated in gradient ethanol (100%, 90%, 70%, and 50% at 2 min each) before rinsing for 5 min in deionized water. The slides were then submerged in hematoxylin (3 min), rinsed with tap water, and then submerged in ammonia water (10 s). After washing in water, the slides were flushed with 1% HCI (10 s). The slides were stained with eosin (2 min) and rinsed in tap water before dehydrating in gradient ethanol (50%, 70%, 90%, and 100% for 2 min each) and then in xylene (2 × 5 min). The slides were mounted in DPX mountant for histology (Sigma-Aldrich) and assessed with whole-slide imaging (NanoZoomer-XR, Hamanatsu, Japan). The slides were quantified in a blind fashion for the percentage of tubules with normal spermatogenesis as outlined by Xie et al.[40] with 200 seminiferous tubules in four sections assessed for each mouse.

Natural mating

After 20.5 weeks on the diets, the founder males were given the opportunity to mate with two normal-weight 10-week-old C57BL6 females over two mating periods of 4 days (3 days apart). The female mice were exposed to the founder males during the dark cycle only and separated and maintained on standard chow during the light cycle. During the mating period, males allocated to HFD + metformin treatment were given plain water during the dark cycle to limit the exposure of metformin to females. During the light cycle, these males were returned to their normal metformin treatments as above. Successful mating was assessed the following morning by the presence of a vaginal plug. After successful mating, the female mice were group housed until day 15 of pregnancy, where they were studied postmortem to determine litter size, fetal weights and lengths, placental weights, and fetal morphology for day 15 of growth as defined by Wahlsten and Wainwright.[41]

Statistics

All data were expressed as mean ± standard error mean (s.e.m.) and checked for normality using the Kolmogorov–Smirnov test and equal variance using Levene's test. Statistical analysis was performed in SPSS (SPSS version 18, SPSS Inc., Chicago, IL, USA), with AUC and AAC calculated in GraphPad Prism (GraphPad Software version 6, San Diego, CA, USA). $P < 0.05$ was considered statistically significant, and the statistical analysis was accepted if the power of the model was ≥80%. Founder body composition, hormones and metabolites, testicular histology, glucose and insulin tolerance testing (AUC/AAC), and all sperm measures were analyzed by a one-way ANOVA with a Tukey HSD post hoc test to determine differences between groups when more than two groups were present. Founder weekly weights were analyzed by a repeated-measure ANOVA, comparing means across time (repeated observation) and across groups. Individual changes in whole blood glucose concentrations during the glucose and insulin tolerance tests were assessed by a two-way ANOVA with a Tukey HSD post hoc comparing both time and group. Mating rates, pregnancy rates, and fetal morphology (skin, eyes, ears, and limbs) were analyzed using a binomial regression. All litters and fetuses were analyzed. Fetal weights and lengths and placental weights were analyzed using a General Linear Model with a Tukey HSD post hoc test to determine the difference between groups. In the model, father ID and mother ID were included as a random effect to adjust for dependence in results between offspring from the same father and mother and litter size as a fixed variable to compare litter size variations between and within treatments.

RESULTS

Male body composition, glucose and insulin tolerance testing, and plasma metabolites

Initial feeding

During the initial feeding period, males fed with a HFD were heavier from 3 weeks on the diet than males fed a CD (**Supplementary Figure 1a**, $P < 0.01$). This resulted in a 50% increase in weight gained by males fed with a HFD compared with CD-fed males (**Supplementary Figure 1b**, $P < 0.001$). Males fed with a HFD exhibited glucose intolerance as measured by a GTT, compared with males fed with a CD, with increased blood glucose levels at 30, 60, and 120 min postglucose bolus, resulting in a larger AUC (**Supplementary Figure 1c** and **1d**, $P < 0.05$). However, insulin sensitivity was maintained with no differences between males fed with a HFD or a CD for insulin AAC (**Supplementary Figure 1e** and **1f**, $P > 0.05$).

Intervention

During the 6-week interventional period, males fed with either HFD (with or without metformin treatment) or CD maintained their preinterventional body weights (**Supplementary Figure 2a** and **2b**) such that males fed with a HFD (with or without metformin treatment) still displayed a 33% increase in body weight over controls in this 6-week period (**Supplementary Figure 2a** and **2b** and **Table 1**, $P < 0.01$).

Males fed with a HFD remained glucose intolerant compared with CD-fed males (**Supplementary Figure 2c** and **2d**, $P < 0.05$). However, metformin treatment improved glucose tolerance compared with males fed a HFD (**Supplementary Figure 2c** and **2d**, $P < 0.05$), with reduced glucose levels 30 min after the glucose bolus, resulting in a smaller AUC (**Supplementary Figure 2c** and **2d**, $P < 0.05$), although this did not improve to the same levels of those of CD males (**Supplementary Figure 2c** and **2d**, $P = 0.06$). There was no effect of metformin treatment on insulin sensitivity as measured by an ITT, with all groups displaying similar glucose responses after the insulin bolus and calculated AAC (**Supplementary Figure 2e** and **2f**, $P > 0.05$).

Males fed with a HFD (with or without metformin treatment) had increased adiposity depots as a percentage of total body weight (renal, dorsal, omental, gonadal, and their sum), increased liver and spleen weights both in grams and a percentage of total body weight, and increased pancreas and kidney weights in grams compared with males fed with a CD (**Table 1**, $P < 0.05$). HFD + metformin-treated males' kidney weights as a percentage of body weight reduced compared with that of males fed with a CD (**Table 1**, $P < 0.05$), with males fed with a HFD not different from either group (**Table 1**, $P > 0.05$).

Males fed with a HFD had increased fasting plasma concentrations of glucose, insulin, and c-peptide, with a higher HOMA-IR index compared with males fed with a CD (**Table 1**, $P < 0.05$). Although treating males fed with a HFD with metformin did not alter the plasma concentrations of glucose, c-peptide, and insulin compared with males fed with a HFD or those fed with a CD (**Table 1**, $P > 0.05$), the HOMA-IR index was reduced compared with males fed with a HFD (**Table 1**, $P < 0.05$), similar to the levels of those males fed with a CD (**Table 1**, $P < 0.05$).

Males fed with a HFD had increased plasma concentrations of cholesterol, triglycerides, LDLs, HDLs, leptin, and CRP, compared with males fed with a CD, and metformin treatment did not alter these parameters (**Table 1**, $P < 0.05$).

Table 1: The effect of a high-fat diet with metformin treatment on body composition and plasma metabolites

	Control diet	High-fat diet	High-fat diet + metformin
Total body weight (g)	29.8±0.6[a]	40.2±2.2[b]	40.6±0.7[b]
Postmortem body composition			
Gonadal adiposity (%)	2.58±0.16[a]	4.86±0.21[b]	4.05±0.24[b]
Omental adiposity (%)	0.75±0.09[a]	1.55±0.23[b]	1.43±0.14[b]
Renal adiposity (%)	0.99±0.09[a]	2.03±0.21[b]	2.11±0.10[b]
Dorsal adiposity (%)	0.68±0.05[a]	1.13±0.06[b]	1.24±0.07[b]
Total adiposity (%)	5.02±0.34[a]	9.57±0.60[b]	9.69±0.41[b]
Liver (g)	1.38±0.06[a]	2.61±0.32[b]	2.68±0.17[b]
Liver (%)	4.62±0.12[a]	6.37±0.56[b]	6.59±0.41[b]
Pancreas (g)	0.17±0.01[a]	0.24±0.02[b]	0.21±0.02[a,b]
Pancreas (%)	0.57±0.02	0.55±0.03	0.52±0.03
Kidneys (g)	0.40±0.02[a]	0.50±0.03[b]	0.49±0.02[b]
Kidneys (%)	1.32±0.03[a]	1.24±0.04[a,b]	1.20±0.03[b]
Spleen (g)	0.051±0.003[a]	0.105±0.009[b]	0.098±0.011[b]
Spleen (%)	0.17±0.01[a]	0.26±0.02[b]	0.24±0.03[b]
Metabolites			
Glucose (mmol l⁻¹)	14.9±1.1[a]	19.6±2.0[b]	16.0±0.6[a, b]
Insulin (pg ml⁻¹)	430.8±163.4[a]	2164.8±467.8[b]	1568.9±531.8[a,b]
C-peptide (pg ml⁻¹)	836.2±215.9[a]	1549.7±294.9[b]	1075.4±119.8[a,b]
HOMA-IR	3.27±1.34[a]	9.81±0.63[b]	6.25±1.02[a]
Cholesterol (mmol l⁻¹)	3.84±0.20[a]	5.98±0.62[b]	7.20±0.64[b]
Triglycerides (mmol l⁻¹)	0.29±0.02[a]	0.40±0.02[b]	0.41±0.03[b]
Low-density lipoprotein (mmol l⁻¹)	0.69±0.06[a]	1.92±0.36[b]	2.35±0.37[b]
High-density lipoprotein (mmol l⁻¹)	3.58±0.20[a]	5.74±0.60[b]	5.91±0.53[b]
C-reactive protein (µg ml⁻¹)	13.2±0.4[a]	15.1±0.7[b]	16.5±0.8[b]
Leptin (pg ml⁻¹)	543.9±169.3[a]	3574.6±618.4[b]	3810.6±382.4[b]

Data are expressed as mean±s.e.m. Data are representative of $n=9$ control diet, $n=8$ high-fat diet and $n=8$ high-fat diet + metformin males. Within rows, different letters (a and b) denote statistical significance at $P<0.05$, with values bearing the same superscript not significantly different. HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; s.e.m.: standard error of the mean

Testicular morphology, WHO sperm parameters, and sperm binding to MII oocyte

There was no effect of HFD feeding (with or without metformin treatment) on testicular weight in grams (**Table 2**, $P > 0.05$), although seminal vesicle weights in grams where increased in males fed only with a HFD compared with those of CD-fed males (**Table 2**, $P < 0.05$). There was no effect of diet (CD *vs* HFD) or metformin treatment on plasma circulating testosterone concentrations between males (**Table 2**, $P > 0.05$).

Assessment of testicular histology demonstrated that males fed with a HFD had reduced numbers of morphologically normal seminiferous tubules compared with HFD males treated with metformin (**Figure 1a**, $P < 0.05$). In addition, males fed with a HFD had an increased percentage of tubules with a degrading seminiferous epithelium compared both with males fed with a CD and HFD males treated with metformin (**Figure 1a**, $P < 0.05$). Thus, the treatment of HFD-fed males with metformin improved seminiferous tubule structure compared with males fed with a HFD (**Figure 1a–1c**, $P < 0.05$).

Males fed with a HFD had a reduced proportion of progressively motile spermatozoa and an increased proportion of immotile spermatozoa compared with both males fed with a CD and HFD males treated with metformin (**Table 3**, $P < 0.05$). With males fed a HFD with metformin treatment having sperm motility levels similar to that of CD-fed males (**Table 3**, $P < 0.05$), there was no effect of treatment group on sperm morphology (**Table 3**, $P > 0.05$).

Although there was no observed effect on the number of motile spermatozoa bound to the zona pellucida of MII oocytes between CD-fed and HFD-fed males (**Table 3**, $P > 0.05$), the treatment of HFD males with metformin increased sperm binding compared with HFD-fed males (**Table 3**, $P < 0.05$).
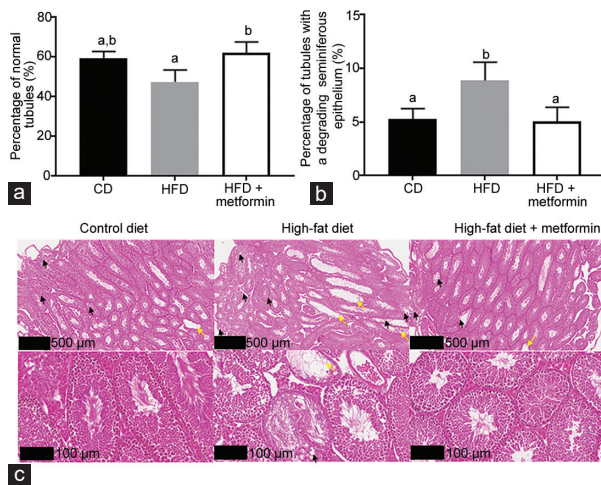
Figure 1: The effect of high-fat diet with metformin treatment on testicular morphology. (**a**) Percentage of normal seminiferous tubules. (**b**) Percentage of tubules that displayed a degrading seminiferous epithelium. (**c**) Representative images of testicular morphology at different magnifications. Scale bars in the top row represent 500 µm, while scale bars in the bottom row represent 100 µm. Black arrows point to tubules containing vacuoles and yellow arrows represent tubules with a degrading seminiferous epithelium. Data are expressed as mean ± s.e.m. Data are representative of $n = 8$ control diet, $n = 7$ high-fat diet and $n = 7$ high-fat diet + metformin males. a and b letters denote significance at $P < 0.05$. Figures bearing the same superscript letter are not significantly different. CD: control diet; HFD: high-fat diet; s.e.m.: standard error of the mean.

Sperm ROS concentrations and oxidative DNA damage

Males fed with a HFD had increased sperm intracellular (DCFDA) ROS concentrations, sperm superoxide (MSR) concentrations, and oxidative sperm DNA damage compared with CD-fed males (**Figure 2a–2c**, $P < 0.05$). HFD-fed males treated with metformin had reduced sperm intracellular ROS concentrations and reduced oxidative DNA damage (8OHdG) compared with males fed with a HFD (**Figure 2a** and **2c**, $P < 0.05$), with concentrations restored back to CD-fed males. Metformin treatment did not alter sperm superoxide (MSR) concentrations, with levels similar to those of CD- and HFD-fed males (**Figure 2b**, $P > 0.05$). Mitochondrial membrane potential, as assessed by the ratio of red-to-green fluorescence of JC-1 in spermatozoa, was not altered in any diet/treatment group (**Figure 2d**, $P > 0.05$).

Mating and fetal outcomes

Males fed with a HFD (with or without metformin treatment) had reduced mating rates compared with that of CD-fed males (**Table 4**, $P < 0.05$), with no differences in the number of mated females that became pregnant between treatment groups (**Table 4**). Males fed with a HFD + metformin treatment had increased litter sizes compared with both HFD and CD-fed males (**Table 4**, $P < 0.05$).

Similar to previous studies, fetuses born to HFD-fed males had reduced day-15 fetal weights and lengths compared with fetus born to CD-fed males (**Table 4**, $P < 0.05$). Treatment of males fed a HFD + metformin increased both fetal weights and lengths compared with those fetuses born to HFD-fed males (**Table 4**, $P < 0.05$), with fetal lengths restored to those of fetuses born to CD-fed males (**Table 4**). HFD-fed males treated with metformin had more fetuses on day 15 with on-time ear development (distinct pinna flap almost over the meatus)

Table 2: The effect of a high-fat diet with metformin treatment on reproductive body composition and plasma testosterone

Reproductive body composition	Control diet	High-fat diet	High-fat diet + metformin
Right testis (g)	0.077±0.003	0.085±0.003	0.082±0.003
Left testis (g)	0.076±0.003	0.086±0.002	0.084±0.003
Seminal vesicles (g)	0.36±0.01[a]	0.44±0.02[b]	0.42±0.01[a,b]
Testosterone (ng ml^{-1})	0.37±0.13	0.31±0.15	0.56±0.20

Data are expressed as mean±s.e.m. Data are representative of $n=9$ control diet, $n=8$ high-fat diet and $n=8$ high-fat diet + metformin males. Within rows, different letters (a and b) denote statistical significance at $P<0.05$, with values bearing the same superscript not significantly different. s.e.m.: standard error of the mean

Table 3: The effect of a high-fat diet with metformin treatment on WHO sperm parameters and ability of sperm to bind to an MII oocyte

Sperm quality	Control diet	High-fat diet	High-fat diet + metformin
Motility			
Progressive motility (%)	33.6±2.3[a]	11.0±2.4[b]	28.3±2.4[a]
Nonprogressive motility (%)	45.5±2.4	51.3±2.5	48.1±2.5
Immotile (%)	20.7±2.2[a]	37.6±2.4[b]	23.5±2.3[a]
Morphology			
Total normal morphology (%)	44.4±1.9	40.3±2.1	44.3±2.0
Abnormal head morphology (%)	19.0±2.4	18.3±2.6	17.7±2.4
Abnormal tail morphology (%)	35.6±3.1	41.4±3.3	39.2±3.1
Sperm function			
Number of spermatozoa bound to an MII oocyte	20.6±0.8[a,b]	17.2±0.7[b]	22.9±0.9[a]

Data are expressed as mean±s.e.m. Data are representative of $n=1800$ sperm from $n=9$ control diet, $n=1600$ sperm from $n=8$ high-fat diet and $n=8$ high-fat diet + metformin males. Within rows, different letters (a and b) denote statistical significance at $P<0.05$, with values bearing the same superscript not significantly different. s.e.m.: standard error of the mean

compared with fetuses born to HFD-fed males, which were delayed in development (**Table 4**, P < 0.05). There was no effect of treatment groups on on-time skin, eye, or limb development (**Table 4**, P < 0.05), with all treatment groups having delayed eye development (all eyes are still almond shaped with no evidence of closing) and skin development (no evidence of wrinkling on the abdomen) compared with *in-vivo* hybrid fetuses as described in the original article.[41]
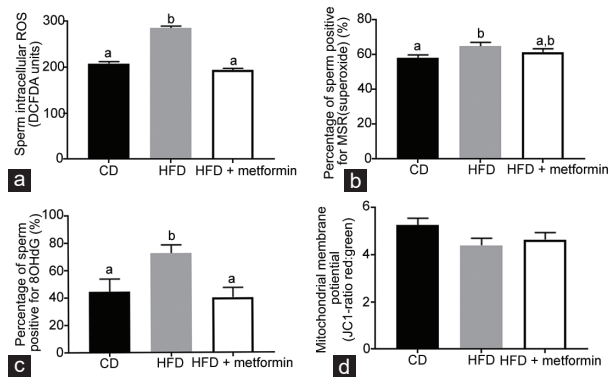
DISCUSSION

Male obesity and associated metabolic syndrome is increasingly prevalent in males attending assisted reproductive technologies,[3] suggesting a link between subfertility and male obesity. While it has been shown that diet and exercise lifestyle interventions in both humans and animal models have positive effects on WHO semen parameters, sperm DNA damage reduction, embryo development and health, pregnancy establishment, and offspring outcomes,[8,9,19–23]

Figure 2: The effect of high-fat diet with metformin treatment on sperm ROS concentrations, oxidative DNA damage, and mitochondrial membrane potential. (**a**) Intracellular sperm ROS generation (DCFDA). (**b**) Percentage of spermatozoa positive for MitoSox Red staining (mitochondrial superoxide). (**c**) Percentage of spermatozoa positive for 8OHdG staining (oxidative DNA damage). (**d**) The ratio of red-to-green JC-1 fluorescents in spermatozoa (mitochondrial membrane potential). Data are expressed as mean ± s.e.m. Data are representative of n = 9 control diet, n = 8 high-fat diet and n = 8 high-fat diet + metformin males. a and b letters denote significance at P < 0.05. Figures bearing the same superscript letter are not significantly different. ROS: reactive oxygen species; 8OHdG: 8-hydroxy-2'-deoxyguanosine; CD: control diet; HFD: high-fat diet; s.e.m.: standard error of the mean.

compliance with these types of interventions in humans is often low. Therefore, it is imperative to uncover alternative interventions that can be better complied with. Little is known about whether pharmacological interventions can also improve sperm function and fertility in obese men. Using metformin to target glucose control, a comorbidity frequently dysregulated in male obesity, we have been able to increase litter sizes and fetal weights and lengths and restore oxidative sperm DNA damage, testicular morphology, and sperm function, all without altering adiposity.

Metformin is a common clinical intervention for the treatment of type II diabetes, poly cystic ovaries, diabetes prevention and gestational diabetes as it is well known for its capacity to lower blood glucose.[24] It has been previously shown to improve testicular and sperm function as well as fertility in other male subfertility models.[25–27] Treating high-fat diet-fed males with metformin in this study increased glucose sensitivity and reduced HOMA-IR concentrations compared with high-fat diet-fed males without metformin. This was likely due to metformin's main mode of action which is to inhibit liver gluconeogenesis and increase insulin-mediated glucose uptake in skeletal muscle.[24] Metformin can reportedly cause a decrease in body weight owing to decreased food intake[42] and reduced cardiovascular risk markers including lipids and inflammatory profiles in mice and humans.[43,44] However, these markers were not altered in the metformin-treated males, with treated males still displaying increased body weight, adiposity, cholesterol, and CRP. Differences between study designs including the length of treatment (ours was relatively short at only 6 weeks), species differences, and provision of the drug (in the drinking water as opposed to some studies which use intravenous injection) are likely explanations for the differences. Further studies are required to determine if increasing dosage time may improve some of these other cardiometabolic risk factors in mouse.

As shown in a previous study,[45] this study showed that metformin treatment in high-fat diet-fed males increased normal testicular morphology and decreased the number of tubules that displayed a degrading seminiferous epithelium. One proposed mechanism for the disruption of testicular morphology from high-fat diet feeding is related to the metabolic balance established between Sertoli cells and developing germ cells.[46] In normal functioning testes, Sertoli cells take up glucose from the interstitial space via high-affinity glucose transporters, with the majority of the glucose converted to pyruvate and then acetyl-CoA. This then enters the mitochondria of Sertoli

Table 4: The effect of a high-fat diet with metformin treatment on mating rates and day-15 fetal development

Fetal development	Control diet	High-fat diet	High-fat diet + metformin
Mated, % (n/total)	77.8 (14/18)[a]	43.8 (7/16)[b]	31.3 (5/16)[b]
Pregnant, % (n/total)	85.7 (12/14)	85.7 (6/7)	100.0 (5/5)
Litter size (n)	7.4±0.3[a]	7.1±0.6[a]	8.7±0.5[b]
Fetal weight (mg)	244±2[a]	216±3[b]	227±3[c]
Fetal length (mm)	11.58±0.06[a]	11.18±0.07[b]	11.42±0.08[a]
Placental weight (mm)	98±1	96±2	99±2
On-time fetal morphological characteristics for day 15, % (n/total)			
Skin features	0 (0/89)	0 (0/42)	0 (0/43)
Limb features	98.8 (88/89)	97.6 (41/42)	100 (43/43)
Eye features	0 (0/89)	0 (0/42)	0 (0/43)
Ear features	98.8 (88/89)[a,b]	92.8 (39/42)[a]	100 (43/43)[b]

Data are expressed as mean±s.e.m. n=9 control diet, n=8 high-fat diet, and n=8 high-fat diet + metformin males had the opportunity to mate with n=18, n=16, and n=16 fertile females, respectively, over two rounds of natural mating. Within rows, different letters (a and b) denote statistical significance at P<0.05, with values bearing the same superscript not significantly different. Percentage of mated was calculated from the number of females that displayed a copulation plug. Percentage of pregnant was calculated from the number of mated females that had fetus. On-time fetal morphology for day 15 was defined as per Wahlsten and Wainwright.[41] Skin features must include follicles on head and abdomen wrinkled. Limb features must have digits splayed with clear phalanges on F and digits separated on H. Eye features include almond shape and beginning to close, and ear features include distinct pinna flap almost over the meatus. All fetuses and litters were analyzed, n=89 control-diet fetus, n=42 high-fat diet fetus, and n=43 high-fat diet+metformin fetus were assessed. s.e.m.: standard error of the mean

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cells to be used in the citric acid (TCA) cycle or made available to the germ cells as fuel for ATP production.[47] Given that obesity is associated with high circulating glucose concentrations, it is likely that more glucose has been transported into the Sertoli cells from the interstitial space, causing the metabolic balance of the Sertoli cells to become disrupted. This may result in more ROS production as a by-product of this perturbed metabolic profile, contributing to the structural and morphological changes seen in the testes of high-fat diet-fed males.[48] This appears likely, as obesity increases ROS concentrations in both testicular tissue and spermatozoa.[49,50] While metformin treatment of high-fat diet rodents with an additional low-fat diet change improves ROS-mediated antioxidant capabilities of the testes,[51] it also reduces oxidative stress and apoptotic markers (malondialdehyde and caspase-3, catalase, superoxide dismutase, and glutathione peroxidase) in testicular tissue after testicular torsion in male rats.[26] Therefore, metformin treatment of high-fat diet-fed male mice likely restores testicular morphology through similar mechanisms.

Metformin treatment of males fed with a high-fat diet was able to reverse the perturbed sperm function observed in male obesity, with increases in progressive sperm motility and improvements in the ability of spermatozoa to bind to and fuse with the zona pellucida of an oocyte. Both these sperm functions are required for successful fertilization and subsequent pregnancy. Metformin exerts its effect mainly through 5' AMP-activated protein kinase (AMPK)-dependent mechanisms by the inhibition of mitochondrial respiration.[52] Sperm energy homeostasis is heavily regulated by AMPK, with its associated pathways important for many sperm functions, including motility, acrosome reaction, and fertilization.[53] Evidence of the activation of AMPK-dependent pathways by metformin has been previously shown in chicken spermatozoa, with increases in sperm production of ATP and lactate coinciding with increases in progressive sperm motility after incubation *in vitro* with metformin.[54] In addition, we have previously shown that spermatozoa from high-fat diet-fed obese mice have a reduced glucose uptake, an AMPK-regulated metabolite.[19,55] Therefore, it is likely that the improvements seen in sperm motility and sperm binding from metformin treatment were due to the activation of AMPK-dependent pathways in reproductive tissues.

In addition to sperm parameters, metformin treatment of high-fat diet-fed males was able to restore intracellular sperm ROS concentrations and oxidative DNA damage. Metformin is known for its antioxidant and anti-inflammatory properties.[56] It has been shown to work both as ROS scavenger *in vitro*[57] as well as altering ROS production at the cellular level.[24] One of the primary cellular targets of the action of metformin is inhibition of complex I of the mitochondrial electron transport chain, causing a decrease in energy production, through switching on catabolic pathways for the generation of ATP and switching off anabolic-consuming ATP and ROS-generating pathways.[24] While physiological levels of ROS are necessary for spermatogenesis and postejaculation maturation including capacitation and hyperactivation,[58] these processes quickly become impaired if the cells enter a state of oxidative stress. Spermatozoa are highly susceptible to oxidative damage owing to the lack of cytoplasmic scavenging enzymes and high levels of polyunsaturated fatty acids in their plasma membranes.[59] High concentrations of intracellular sperm ROS are associated with infertility and are a mediator in paternal programming.[60] Therefore, metformin appears an effective treatment for reducing sperm ROS concentrations in high-fat diet-fed male mice.

A systematic review with meta-analysis has shown that male obesity reduces time to conception and overall fecundity.[3] These effects have been attributed to premature ejaculation, decreased libido, erectile dysfunction, lower testosterone levels, and higher estrogen levels seen in obese men.[61] In this study, metformin treatment of high-fat diet-fed mice did not improve mating rates. Plasma testosterone concentrations, which relate to libido, were not altered across treatment groups. Therefore, the reductions in mating rates may have been more related to the size differences in high-fat diet-fed males (increased central adiposity) compared with controls and the ability to chase and mount fertile females.

Metformin treatment of high-fat diet-fed obese male mice increased fetal weights and lengths and litter sizes, with measures almost restored to those of controls. This is similar to the findings of metformin-treated streptozotocin-induced diabetic male rats, where litter sizes and fetal weights were restored to control levels.[25] Male obesity in both humans and animal models is associated with reduced fetal weights and increased susceptibility of neurological developmental problems and increased metabolic syndrome risk in subsequent offspring.[6,7,11–13,62,63] It is probable that short-term metformin treatment in obese males would also improve offspring health. Therefore, translation of these studies to obese men, particularly in those with aberrant glucose control, appears warranted.

As in our exercise interventional study on mice fed with a high-fat diet,[19,21] metformin treatment was able to improve glucose tolerance and restore sperm function without reducing adiposity. Similar findings have been found in humans, where it was found that 6 months of metformin treatment to 45 subfertile male patients with metabolic syndrome improved insulin resistance as measured by HOMA, without a reduction in waist circumference or body mass index, subsequently increasing sperm count, motility, and normal morphology.[64] Given that metformin's main mode of action is lowering glucose, these data suggest that hyperglycemia may be an intermediate between male obesity and altered fertility independent of adiposity.

CONCLUSION

This study showed that a pharmacological intervention targeting glucose control (metformin) in male mice fed with a high-fat diet to induce obesity, restored sperm and testicular function, reduced sperm oxidative stress and increased litter sizes and fetal weights, all without a change in adiposity. Therefore, short-term metformin treatment could be a potential intervention for the treatment of obesity-induced subfertility in men without a dramatic change to lifestyle. Further studies in humans are warranted.

AUTHOR CONTRIBUTIONS

NOM and ML designed and performed the study. NOM analyzed and interpreted the data. NOM wrote the manuscript. ML edited and approved the final manuscript. Both authors read and approved the final manuscript.

COMPETING INTERESTS

ML and NOM are paid employees of the Monash IVF group. NOM is the recipient of an NHMRC Early Career Fellowship.

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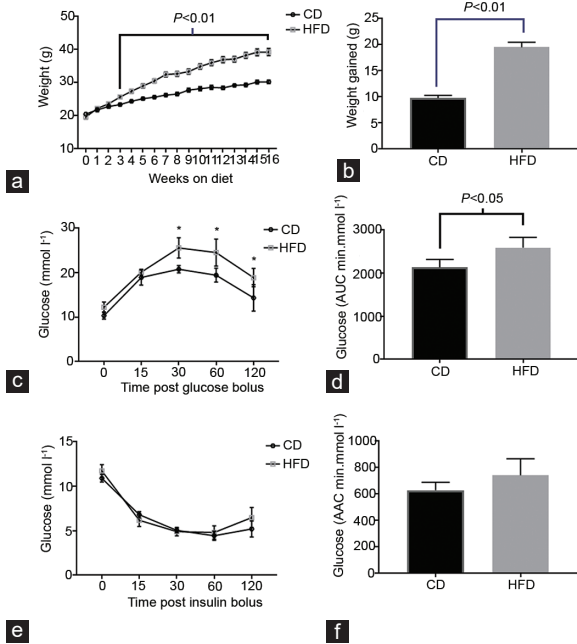
Supplementary Figure 1: The effect of a high-fat diet on weight gain and glucose and insulin tolerance. (**a**) Weekly weight gained during the initial feeding period. (**b**) Total weight gained during the initial feeding period. (**c**) Glucose tolerance as assessed by GTT (2 g kg^{-1}) at 15 weeks of diet feeding. (**d**) Glucose AUC (min.mmol l^{-1}) during GTT after the initial feeding period. (**e**) Insulin tolerance as assessed by ITT (1.0 IU) at 16 weeks of diet. (**f**) Glucose AAC (min.mmol l^{-1}) during ITT after the initial feeding period. Data are expressed as mean ± s.e.m. Data are representative of $n = 9$ CD males and $n = 16$ HFD males. *Denotes statistically significant differences at $P < 0.05$ between control diet and high-fat diet at their corresponding time points during the GTT. HFD: high-fat diet; GTT: glucose tolerance test; AUC: area under the curve; ITT: insulin tolerance test; AAC: area above the curve; s.e.m.: standard error of the mean; CD: control diet.
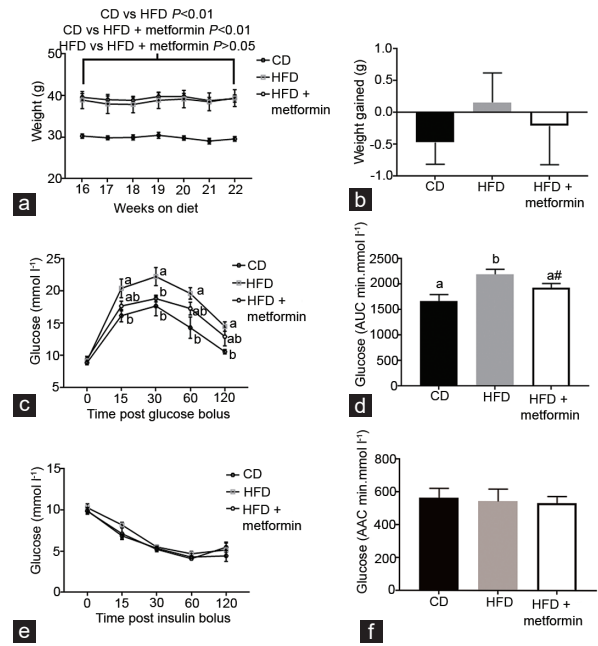
Supplementary Figure 2: The effect of high-fat diet with metformin treatment on weight gain and glucose and insulin tolerance. (**a**) Weekly weight gained during the interventional period. (**b**) Total weight gained during the interventional period. (**c**) Glucose tolerance as assessed by GTT (2 g kg^{-1}) at 21 weeks of diet feeding. (**d**) Glucose AUC (min.mmol l^{-1}) during GTT after the interventional period. (**e**) Insulin tolerance as assessed by ITT (1.0 IU) at 22 weeks of diet feeding and (**f**) glucose AAC (min.mmol l^{-1}) during ITT after the interventional period. Data are expressed as mean ± s.e.m. Data are representative of $n = 9$ control diet males and $n = 8$ high-fat diet and high-fat diet + metformin males. Different letters denote statistical significance at $P < 0.05$. Figures bearing the same superscript are not significantly different. #Difference between high-fat diet + metformin and control diet males at $P = 0.06$. GTT: glucose tolerance test; AUC: area under the curve; ITT: insulin tolerance test; AAC: area above the curve; s.e.m.: standard error of the mean.