Altered glucose metabolism in mouse and humans conceived by in-vitro fertilization (IVF)

Altered glucose metabolism in IVF offspring

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Abstract (200 words)

In-vitro fertilization (IVF) may influence the metabolic health of children. However, in humans, it is difficult to separate out the relative contributions of genetics, environment, or the process of IVF, which includes ovarian stimulation and embryo culture. Therefore, we examined glucose metabolism in young adult humans and in adult male C57BL/6J mice conceived by IVF versus naturally, under energy balanced and high-fat overfeeding conditions. In humans, peripheral insulin sensitivity, as assessed by hyperinsulinemic-euglycemic clamp (80mU/m²/min), was lower in IVF (n=14) versus controls (n=20) after 3 days of an energy-balanced diet (30% fat). In response to 3-days of overfeeding (+1250 kcal/day, 45% fat), there was a greater increase in systolic blood pressure in IVF versus controls (P=0.02). Mice conceived following either ovarian stimulation alone or IVF weighed significantly less at birth versus controls (P<0.01). However, only mice conceived by IVF displayed increased fasting glucose, impaired glucose tolerance and reduced insulin-stimulated Akt phosphorylation in liver following 8 weeks of either chow or high-fat diet (60% fat). Thus, ovarian stimulation impaired fetal growth in mouse, but only embryo culture resulted in changes in glucose metabolism that may increase the risk of developing metabolic diseases later in life, and in both mouse and humans.

Clinical trial registry no.: NCT01230632; ClinicalTrials.gov.
Assisted reproduction technologies, mostly in-vitro fertilization (IVF) and intracytoplasmic sperm injection, are increasingly used to treat infertility with the number of children now in excess of 5 million worldwide (1). Of concern, is emerging evidence to suggest that IVF children may be at an increased risk of developing metabolic and cardiovascular diseases (2). In particular, studies have reported that IVF children have increased fasting glucose levels, blood pressure, triglycerides, adiposity, and inflammatory biomarkers, as well as systemic and pulmonary vascular dysfunction (3-7). To date, it has not been tested whether any differences in insulin sensitivity are apparent between these groups, which may underlie many of these differences. Moreover, it is unclear if increased risk factors observed in IVF children are related to dietary patterns, the underlying genetics of the parents, environmental factors, or the treatment procedures per se.

Animal models may provide evidence of biological plausibility and potential mechanisms. Impaired glucose tolerance, increased systolic blood pressure and body fat, altered fatty acid composition in liver and adipose tissue, endothelial dysfunction and increased stiffness, and shorter life span have all been reported in IVF conceived mouse offspring (8-12). These studies suggest that the procedure of IVF itself may increase metabolic risk. However, these studies have rarely controlled for litter size or the maternal environment between groups, which may also impact outcomes. It is also unclear whether any differences in outcomes are due to the process of ovarian stimulation with high doses of gonadotropins prior to oocyte collection and/or the process of in vitro embryo culture.

The aim of this study was to examine insulin sensitivity and metabolic risk factors in young adults conceived by IVF or natural conception (NC) following an energy balanced diet (30% fat) and following 3-days of high-fat overfeeding challenge (45% fat). In parallel, a study in C57BL/6J mice directly compared key metabolic factors in adult male offspring that were generated by IVF versus those that were naturally conceived. Importantly, mice conceived
following ovarian stimulation alone were also examined, allowing us to separate out the
effects of ovarian stimulation versus embryo culture.

Research design and methods

Human study participants

Young adults conceived by IVF were recruited by advertising in local newspapers and a
University campus or from a database of IVF birth records in South Australia (ACN
008123466 Pty Ltd) and were matched by sex and BMI to naturally conceived individuals.
Participants were excluded if they were not of normal birth weight, reported any significant
medical conditions or took any medications that may alter glucose or lipid metabolism (eg.
Metformin), or had first degree relatives with type 2 diabetes or cardiovascular disease, or if
they smoked or drank >140g of alcohol/week. All parents were Caucasian, except two sets of
parents were Chinese (one IVF male and one NC male).

Human study design

Volunteers visited the clinical research facility for screening to determine eligibility, which
included medical history, blood lipids and fasting glucose. Thirty-four individuals were
recruited. Three IVF individuals were twins and one naturally conceived individual was a
twin. Body composition was measured by dual energy x-ray absorptiometry (Lunar DPX-
Lunar Radiation, Madison, USA). Female participants were tested in their follicular phase of
menstrual cycle. Two females from each group were taking oral contraception pill. Three
females (NC 2, IVF 1) did not undergo repeat assessments following the overfeeding. The
study protocol was approved by the Research Ethics Committee of Royal Adelaide Hospital.
Informed written consent was obtained from all participants before commencement of the
study.
Prior to metabolic testing at baseline, estimated energy requirements were calculated and individual menus were planned by a trained dietitian, as previously described (13). From day -3 to day 0, individuals were fed an energy balanced diet (30% fat, 15% protein, and 55% carbohydrate). Following baseline metabolic testing, individuals were switched to an overfeeding diet (+1250kcal/d) with a nutrient composition of 45% fat, 15% protein, and 40% carbohydrate for 3 days before metabolic assessments were repeated. Participants were provided with all foods and completed checklists reporting foods consumed, which were reviewed at each metabolic testing visit. Energy intake was comparable between groups (Supplementary Table1).

**Metabolic tests in participants**

Participants attended the clinical research facility at 8am after a 12-hour overnight fast. The procedures at the two visits were identical. Weight, height and blood pressure were measured in a hospital gown after voiding. First phase insulin secretion was assessed by an intravenous glucose tolerance test. After the fasting blood collection, a bolus dose of 25% glucose (0.3 g/kg with a maximum of 30 g) was injected into the antecubital vein within 1 minute. Blood was collected at 1, 3, 4, 5, 6, 7, 8, 10 minutes after glucose infusion. Insulin sensitivity was then measured using a 2-hour hyperinsulinemic–euglycemic clamp (80 mU/m² body surface/min) as described previously (14).

**Biochemical analysis**

Glucose was analyzed using a glucose oxidase electrode (YSI Life Sciences). Serum insulin was assayed by radioimmunoassay (Millipore). Blood lipids were examined by photometric assays in the laboratory of SA Pathology, South Australia.
**IVF Mouse Model**

C57BL/6J mice were obtained at 6-weeks of age, and vasectomized (CBA × C57BL/6) F1 male mice were obtained at 8-weeks of age from the Animal Resource Centre (Perth, Western Australia). (CBA × C57BL/6) F1 female mice were obtained at 6-weeks of age from the Laboratory Animal Services (Adelaide, Australia). All mice were maintained on a 12-hour light, 12-hour dark cycle, with standard rodent chow diet (SF06-105, Specialty Feeds) and water available ad libitum. All mice were acclimatized for 2 weeks on chow before experimentation. All experiments were approved by the University of Adelaide Animal Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

**Generating blastocysts**

Mouse blastocysts were generated by natural conception (NC group), or by ovarian hormonal stimulation followed by either mating (OS group) or by IVF and embryo culture (IVF group). To generate pups for the NC group, female C57BL/6J mice were placed with male C57BL/6J mice overnight. For the OS group, female C57BL/6J mice were superovulated with consecutive injections of 7.5 IU equine chorionic gonadotropin (Calbiochem) and 7.5 IU human chorionic gonadotropin (hCG; Calbiochem) administered intraperitoneally 48 hours apart. After injection with hCG, these females were placed with male C57BL/6J mice overnight. The following morning, female mice (NC and OS groups) with the presence of vaginal plugs were considered to be pregnant and three days later, were humanely sacrificed by cervical dislocation. Blastocysts were collected by flushing dissected uteri with prewarmed HEPES-buffered minimal essential media (Invitrogen Australia Pty. Ltd.) supplemented with 5 mg/ml human serum albumin (ART-3001, SAGE® Media). Blastocysts from NC and OS group were placed in Research Cleave media (Cook Medical; Australia) for no more than 1 hour prior to being transferred to uteri of pseudopregnant mice (see below).
For the IVF group, the ovarian stimulation protocol was the same as the OS group. In vitro fertilizations were performed using a modified version of that described previously (15). At 13 hours after injection with hCG, female C57BL/6J mice were humanely killed by cervical dislocation. Cumulus-oocyte complexes from the oviducts were placed in Research Fertilization media (Cook Medical) under paraffin oil (Merck Pty.Ltd.) and incubated in a modular incubator chamber at 37°C in 6% CO₂, 5% O₂, 89% N₂ for 5-6 hours with sperm collected from the cauda epididymis of male C57BL/6J mice that had been previously incubated for 1 hour in Research Fertilization media for sperm capacitation. The putative zygotes were then placed in Research Cleave media and incubated in the modular incubator chamber a further 3 days to the blastocyst stage.

**Blastocyst transfer**

Unstimulated (CBA × C57BL/6) F1 female mice were mated with vasectomized males and those with copulatory plugs the next morning were considered as day 0.5 of pseudopregnancy. At day 2.5 of pseudopregnancy, 7-10 blastocysts were transferred to uteri of each pseudopregnant recipient mouse (7-8 litters per group) anesthetized by i.p. injection of Avertin (0.5 mg/g, Sigma-Aldrich). Analgesia Carprofen (5 mg/kg, Rimadyl ® Pfizer) was injected subcutaneously once after the surgery. All recipient females were fed chow diet (SF06-105, Specialty Feeds).

**Pups and diets**

Pups were born on day 19.5 of pregnancy and weighed weekly and weaned at 3-weeks of age onto chow diet or high-fat diet for 8 weeks. High-fat diet was made in house using the same recipe of D12492 as Research Diets (New Brunswick, NJ) (nutrient composition: 60% fat, 20% protein, and 20% carbohydrate). Only male offspring were used for this study.
Glucose and insulin tolerance tests

At 11 weeks of age, mice were fasted 6 hours and challenged with either an intraperitoneal injection of glucose (2g/kg) or insulin (0.75U/kg). Blood samples were obtained from tail tip for assessment of glucose at 0, 15, 30, 60, 120 minutes with a glucometer (Accu Chek Performa, Roche Diagnostics) and insulin at 0, 30, 60, 120 minutes by ultra-sensitive ELISA (Merck Millipore). One week later, mice were either sacrificed by cervical dislocation, and quadriceps, inguinal fat, epididymal fat and liver were immediately excised, weighed and snap frozen or an insulin stimulation test was performed (n=4/group). For this test, mice were fasted for 6 hours and while under anesthesia (pentobarbital 60 mg/kg ip, Sigma-Aldrich), a sample of liver and one quadriceps muscle were collected and snap frozen. Insulin (1U/kg) was then injected into the inferior cava vein and exactly three minutes later, mice were killed by cervical dislocation, and an additional sample of liver and the contralateral quadriceps muscle was rapidly excised and snap frozen.

Immunoblotting

Liver and quadriceps tissues were lysed and protein concentration was determined by Pierce BCA Kit (Thermo Scientific). Lysates (20 µg protein) were resolved by SDS-PAGE and transferred onto PVDF membranes. Membranes were probed for Akt (Cell Signaling), Phospho-Ser473 Akt (Cell Signaling). All blots were applied with ECF substrate (Amersham) and scanned for fluorescence by the Typhoon Trio+ (Amersham biosciences) following the manufacturer’s instructions. The band intensity was measured using Image J software (the National Institutes of Health, USA).

Quantitative real-time PCR

Total RNA was extracted from liver using Trizol (Invitrogen). cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR was performed as described previously (16) using the TaqMan primers and probes listed in Supplementary...
Table 2. The NormFinder program was used as described previously (17) and Hprt and Ppia out of 7 potential reference genes were identified as the best combination. Data were analyzed using the $2^{-\Delta\Delta CT}$ method.

**Statistical analysis**

Data are shown as mean ± SEM, unless otherwise stated. Data were analyzed statistically with SPSS 20 (SPSS, Chicago, USA). For the human data comparisons, baseline differences between groups were analyzed by Student’s $t$-test, and response to diet intervention was assessed using repeated measures ANOVA and Bonferroni post hoc analysis and an intention-to-treat approach without carrying forward data on the three dropouts with gender and group in the model. Genders were combined for analysis since no differences in response were detected by gender. For animal studies, single comparisons were performed with Student’s $t$-test or two-way ANOVA, whereas time-courses were analyzed by repeated-measures ANOVA and Bonferroni post hoc analysis. When required, nonparametric tests (Mann-Whitney U test or Kruskal-Wallis test) were used as indicated. Differences were considered statistically significant at $P<0.05$.

**Results**

**Decreased insulin sensitivity in IVF conceived humans**

There was no significant difference in Z-score of birth weight adjusted for gestational age, maternal age and gender (-0.11 (0.87) for IVF group and 0.08 (1.1) for NC group ($P=0.23$)) or between groups in terms of length of gestation or parental characteristics during pregnancy including smoking, alcohol consumption and health (data not shown), except mothers undergoing IVF treatment were significantly older than those conceived naturally (34 ± 1 vs 28 ± 1 years, $P=0.001$).
There were no significant differences between groups at baseline with respect to age, weight, BMI, % fat mass, blood pressure, or cholesterol levels (Table 1). Fasting glucose, fasting insulin and HOMA-IR were also not different between groups at baseline, but peripheral insulin sensitivity as measured by the hyperinsulinemic–euglycemic clamp was significantly lower in IVF versus naturally conceived individuals. First phase secretion of insulin (shown by insulin area under the curve 10min, insulin AUC$_{10\text{min}}$) and glucose change (glucose AUC$_{10\text{min}}$) in response to intravenous glucose tolerance test was not different between groups.

**Metabolic consequences of high-fat overfeeding in humans**

Body weight gain in response to 3-days of overfeeding was not significantly different between groups (NC 0.6 ± 0.2 vs IVF 0.7 ± 0.2 Kg). As we have previously observed, fasting glucose, insulin, and thus HOMA-IR were increased significantly in response to 3-days of overfeeding in both groups (fasting glucose, NC 0.06 ± 0.04 vs IVF 0.1 ± 0.05 mmol/L; Insulin, NC 0.4 ± 0.6 vs IVF 2 ± 0.6 µU/ml; and HOMA-IR, NC 0.1 ± 0.1 vs IVF 0.4 ± 0.1 AU; diet effect, P<0.01). The increase in insulin or HOMA IR did not reach statistical significance between IVF versus naturally conceived individuals (both P=0.1). However, there was a greater increase in systolic blood pressure in IVF versus naturally conceived individuals in response to overfeeding (NC 107 ± 2 vs IVF 115 ± 3 mmHg, diet x group interaction P=0.04; post hoc test P=0.02). No other parameters described at baseline were altered following overfeeding, and no other group differences were detected (data not shown).

**Reduced fetal growth in mice conceived by ovarian stimulation and IVF**

Litter size was not different between groups (NC 6.3 ± 0.6, OS 7.4 ± 0.7, IVF 6.9 ± 0.5, P=0.5). Birth weight of IVF pups (1.51 ± 0.03, n=26) and OS pups (1.51 ± 0.02, n=28) was significantly lower than NC pups (1.68 ± 0.03, n=21), and this difference was maintained until 3-weeks of age (Figure 1). Following weaning at 3-weeks of age, body weight remained
significantly lower in OS and IVF mice on both diets. As expected body weight and weight
gain were increased by HFD. However, body weight gain either before weaning or after
weaning was not different between groups.

High-fat diet decreased relative liver weight and increased relative adipose tissue weight, but
there were no differences between IVF and control mice on either diet (Figure 2). However,
the relative weight of inguinal and epididymal fat pads were significantly lower in OS mice
fed a HFD.

**IVF mice display impaired glucose tolerance**

As expected, fasting glucose and insulin were increased by HFD (Figure 3). Interestingly,
IVF mice displayed higher fasting glucose as compared with NC and OS mice, independently
of diet. OS and IVF mice had lower fasting insulin than NC mice, following HFD only. In
response to glucose challenge, IVF mice on both diets had impaired glucose tolerance as
evidenced by increased glucose AUC as compared to NC mice, but the insulin response to
 glucose was not different between groups for chow fed mice (Figure 3). Insulin data was not
available for mice on HFD, but peripheral insulin sensitivity as assessed by intraperitoneal
insulin tolerance (data not shown), and by insulin-stimulated Akt-Ser\(^{473}\) phosphorylation in
muscle was not different between groups fed either chow or HFD. However, reduced Akt-
Ser\(^{473}\) phosphorylation was observed in liver of IVF mice fed chow and HFD (Figure 4).

**Altered hepatic gene expression in IVF mice**

Hepatic expression of gluconeogenesis gene G6pc and mitochondrial biogenesis markers
Cpt1α, Pgc1α and Tfam were not different between groups or diets (Figure 5), and protein
levels of Pgc1α and total OXPHOS were also not altered (data not shown). High-fat diet
increased the expression of glucokinase and decreased the expression of gluconeogenic gene
Pck1 in all groups (Figure 5). Compared with NC mice, the expression of lipogenesis gene Srebfl was increased in IVF mice fed chow or HFD.

Discussion

Suboptimal maternal environment during pregnancy predisposes offspring to chronic diseases later in life (18; 19), with the preimplantation period also emerging as a critical stage for development and later adult health (20; 21). Accumulating evidence suggests that children conceived by IVF may have increased risk of developing metabolic syndrome, type 2 diabetes and cardiovascular disease (3-7). In this study, we observed that IVF adult humans, the majority of whom were normal weight, were more insulin resistant than BMI, sex and aged matched naturally conceived individuals. In the carefully controlled mouse study, we showed that IVF conceived mice displayed hyperglycemia, impaired glucose tolerance and hepatic insulin resistance at both normal and high body weight. Thus, our study supports the hypothesis that IVF alters glucose metabolism and increases the risk of developing metabolic diseases, later in life.

This is the first study to test peripheral insulin sensitivity in IVF young adults by using gold standard assessment with the hyperinsulinemic-euglycemic clamp. We observed reduced peripheral insulin sensitivity in IVF adults, although we did not note any significant differences in fasting glucose or insulin levels, which is similar to observations made in cohorts of young non-diabetic individuals with a strong family history of type 2 diabetes (13; 22). In contrast, some but not all studies have reported increased fasting glucose, triglycerides, blood pressure, or peripheral body fat in IVF children or adolescents (3-5). One study has noted more favorable lipid profiles in prepubertal IVF children with higher high-density lipoprotein levels and lower triglyceride levels (23). Discrepancies between studies may be due to differences in the ages investigated, sample size, sampling of the comparison group,
dietary intake and/or parental characteristics, such as gestational weight gain, maternal and paternal BMI. In this study, maternal age was higher in the IVF cohort, which may alter oocyte quality (24) and may have contributed to the observed effects. Further study is also needed to determine whether reduced insulin sensitivity is related to impaired suppression of hepatic glucose production, and if these differences will be evident in overweight cohorts.

Given the young age of the cohort under investigation, we also examined the metabolic consequences of 3-days of high-fat overfeeding challenge. Typically, overfeeding diets rapidly increase glucose and insulin levels within 3 days (13). Impaired suppression of hepatic glucose production, as assessed by the hyperinsulinemic-euglycemic clamp, is also observed within 5-11 days in young healthy men (25; 26). As noted in this study, longer lengths of time may be necessary to induce peripheral insulin resistance (14; 27). We also observed that 3-days of high-fat overfeeding increased fasting insulin and HOMA-IR, which is reflective of hepatic insulin resistance (28; 29). Interestingly, we have previously identified greater increases in HOMA-IR in response to a similar overfeeding protocol in non-diabetic individuals who have a family history of type 2 diabetes (13), whom have a 1.7-6.1 fold greater risk of developing type 2 diabetes (30). However, this did not reach statistical significance between IVF and naturally conceived individuals in this study. Elevated response of systolic blood pressure in IVF adults was unmasked by a 3-day overfeeding challenge. Consistently, increased blood pressure and vascular stiffness, and endothelial dysfunction have been reported previously in IVF mouse models and children (4; 5; 7; 9; 11). This may be related to insulin, since a large number of clinical studies have confirmed that higher systolic blood pressure is related to increased fasting insulin and insulin resistance, independently of age, BMI, sex, and race (31-33), although we did not observe a correlation between the changes in insulin and blood pressure in this study. IVF mice are also more susceptible to a high-fat diet (50% fat) as evidenced by nearly a 25% shorter life span
compared with naturally conceived mice (11). Together, this data suggests that individuals conceived by IVF may be more sensitive to deleterious consequence of obesogenic environments.

In a previous mouse study, IVF male offspring displayed insulin resistance, but normal glucose response to intraperitoneal glucose, and females displayed impaired glucose tolerance on chow diet at 8 weeks of age (10). However, the genetic background, maternal environment and litter sizes were not controlled in that study. To separate out these potential confounders, and the effects of ovarian stimulation versus embryo culture, we developed an IVF mouse model using inbred C57BL/6J mice. In this study, IVF mice demonstrated impaired glucose metabolism, as evidenced by higher fasting glucose levels, impaired glucose tolerance and impaired hepatic insulin signaling at 12 weeks of age on both chow diet and HFD. Importantly, mice conceived by ovarian stimulation alone did not exhibit any of these differences on either diet compared with controls, indicating that it is the process of embryo culture rather than hormonal stimulation that contributes to impaired glucose metabolism in male offspring. In support of this, others have reported that embryo culture alters blastocyst formation, fetal development and postnatal phenotype, including increased anxiety, poor spatial memory, and elevated systolic blood pressure (9; 34; 35). A recent study demonstrated that IVF also led to vascular dysfunction in mouse and humans (7; 11). Strikingly, this was even observed in IVF mice offspring conceived by transferring 2 cell embryos after just 30 hours of in vitro culture and these differences were transmitted to the next generation (11).

IVF singletons have a higher risk of low birth weight and preterm birth than their non-IVF siblings (36; 37). Similarly, we observed that birth weight was lower in mice conceived either by ovarian stimulation alone or by IVF. This is the reverse of other mouse studies of IVF (10), but in these cases maternal environment was not identical and IVF litters were much smaller.
than naturally conceived litters, issues that were carefully controlled in our study. Our study suggests that ovarian stimulation impairs fetal growth and this is supported by human studies (38-41), and may be related to reductions in oocyte quality and embryo quality (42; 43). In our mouse model, all blastocysts were transferred into unstimulated surrogate recipients with a natural uterine environment, suggesting that any differences in fetal growth were from influencing oocyte and/or embryo development, and were not due to changes in endometrium receptivity. We also observed mice conceived by ovarian stimulation had smaller fat mass gain on a HFD, which may explain the lower fasting insulin observed following HFD in this group. In rodent models, low birth weight is associated with increased risk of type 2 diabetes (44; 45). However we did not observe any metabolic defects in OS mice, who were also born small.

In the present study, IVF mice displayed elevated fasting glucose and impaired glucose tolerance, but no difference in the insulin response to glucose and this, combined with lower fasting insulin on HFD, may be indicative of impaired β-cell function. Alternatively, impaired suppression of basal hepatic glucose production may be responsible both for differences in fasting glycaemia and impaired glucose tolerance. This is supported by our findings of reduced Akt-Ser473 phosphorylation in liver following an insulin stimulation test. Increased gene expression of Srebf1, which is a master regulator of lipogenesis, was also observed in liver obtained from IVF mice, irrespective of diet. Of note, the phenomenon of selective insulin resistance via Akt pathway, but continued sensitivity via Srebf1 pathway, has been reported in other mouse models of type 2 diabetes (46; 47).

In conclusion, IVF conceived human individuals were more insulin resistant and tended to be more susceptible to the metabolic consequences of high-fat overfeeding. Our data in mice are partially supportive of these findings, and further delineate the effects of embryo culture versus ovarian stimulation. These suggest that it is the process of embryo culture itself rather
than genetic and/or environmental differences that contribute to impaired glucose metabolism and that ovarian stimulation impaired fetal growth, at least in mouse. This study highlights an increased risk of developing metabolic and cardiovascular disease in IVF offspring later in life.
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No potential conflicts of interest relevant to this article were reported.

M.C. recruited human subjects, performed the mouse experiments, acquired and analyzed the human and mouse data, wrote the manuscript, critically reviewed the manuscript and approved the final manuscript. L.W. contributed to setting up the mouse model, critically reviewed the manuscript and approved the final manuscript. J.Z. contributed to recruiting human subjects, acquired the data of the human study, critically reviewed the manuscript and approved the final manuscript. F.W. contributed to acquiring data in the mouse study, critically reviewed the manuscript and approved the final manuscript. M.J.D. contributed to recruiting human subjects, critically reviewed the manuscript and approved the final manuscript. G.A.W. contributed to interpretation of the data and helped to draft the manuscript, critically reviewed the manuscript and approved the final manuscript. R.J.N. contributed to recruiting human subjects and interpreting data, critically reviewed the manuscript and approved the final manuscript. R.L.R. designed and supervised the mouse study, interpreted data, critically reviewed and revised the manuscript, and approved the final manuscript. L.K.H conceived, designed and supervised the human and mouse study, interpreted data, critically reviewed and revised the manuscript, and approved the final manuscript. L.K.H is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Data are presented as mean ± SEM. BP, blood pressure; HOMA-IR, homeostasis model of assessment - insulin resistance; AUC, area under the curve; GIR, glucose infusion rate; FFM, fat free mass.
Figure 1 Body weight and weight gain in male mice. (A), * IVF vs NC, P=0.02; ** IVF & OS vs NC, P<0.01. (B), * IVF & OS vs NC, P<0.01; ** Diet effect, P<0.001. (C), fractional weight gain (%) was calculated as: (body weight - birth weight) × 100/ birth weight. (D), weight gain after weaning was calculated as increase from body weight at 3 weeks of age. ** Diet effect, P<0.001.
Figure 2 Tissue weight ratio (normalized to body weight) of liver, inguinal fat and epididymal fat in male mice. ** Diet effect, P<0.001. (B), Group effect, P = 0.002; Group by diet effect, P = 0.004; Post hoc test, * OS vs NC & IVF. P<0.001. (C), Group effect, P = 0.03; Group by diet effect, P = 0.04; Post hoc test,* OS vs NC & IVF. P<0.01.
Figure 3 Fasting glucose (A), fasting insulin (B), and intraperitoneal glucose tolerance tests (C-F) in male mice. ** Diet effect, P<0.001. (A),* IVF vs OS & NC, P<0.001; (B), Group effect, P = 0.004; Group by diet effect, P = 0.007; Post hoc test, * IVF vs NC, P=0.02; OS vs NC, P=0.001; (C), blood glucose following glucose challenge. (D), glucose area under the curve (AUC). AU, arbitrary units. * IVF vs NC, P=0.03. (E), blood insulin following glucose challenge. (F), insulin area under the curve.
Figure 4 Basal and insulin stimulated phosphorylation of Akt Ser\(^{473}\) in liver (A-B) and muscle tissue (C-D) from male mice fed chow or HFD and untreated (-) or treated (+) with insulin for 3 minutes. Left panels show representative sample of one mouse per treatment group. Right panels show quantification of Western blots, as the fold change in pAKT relative to total AKT in response to insulin stimulation from n=3-4 mice per group. (B), * IVF vs NC, P=0.02.
Figure 5 Hepatic gene expression of male mice. White bars represent NC group, grey bars represent OS group and black bars represent IVF group (n=6); (A) * IVF vs NC, P=0.01; (E), ** Diet effect, P=0.01; (G), ** Diet effect, P=0.006.
Supplementary Table 1 Diet diary analysis at baseline and during overfeeding by group

<table>
<thead>
<tr>
<th>Component</th>
<th>NC Baseline</th>
<th>NC Overfeeding</th>
<th>IVF Baseline</th>
<th>IVF Overfeeding</th>
<th>P value</th>
<th>Time</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (Kcal)</td>
<td>2475 ± 340</td>
<td>3808 ± 341</td>
<td>2496 ± 296</td>
<td>3805 ± 288</td>
<td>&lt;0.001</td>
<td>0.9</td>
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<tr>
<td>Fat (g)</td>
<td>82 ± 11</td>
<td>204 ± 14</td>
<td>82 ± 11</td>
<td>201 ± 13</td>
<td>&lt;0.001</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>348 ± 51</td>
<td>356 ± 46</td>
<td>352 ± 43</td>
<td>363 ± 42</td>
<td>&lt;0.001</td>
<td>0.8</td>
<td></td>
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<tr>
<td>Protein (g)</td>
<td>87 ± 13</td>
<td>136 ± 13</td>
<td>89 ± 10</td>
<td>136 ± 8</td>
<td>&lt;0.001</td>
<td>0.9</td>
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</tr>
</tbody>
</table>

Data are presented as mean ± SD.
Supplementary Table 2 TaqMan primers and probes used for gene expression analysis

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<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Reference number</th>
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</thead>
<tbody>
<tr>
<td>Pgc1a</td>
<td>Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha</td>
<td>Mm01208835_m1</td>
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<tr>
<td>Tfam</td>
<td>Mitochondrial transcription factor A</td>
<td>Mm00447485_m1</td>
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<tr>
<td>Cpt1a</td>
<td>Carnitine palmitoyltransferase 1A</td>
<td>Mm01231183_m1</td>
</tr>
<tr>
<td>G6pc</td>
<td>Glucose-6-phosphatase catalytic subunit</td>
<td>Mm00839363_m1</td>
</tr>
<tr>
<td>Gck</td>
<td>Glucokinase</td>
<td>Mm00439129_m1</td>
</tr>
<tr>
<td>Pck1</td>
<td>Phosphoenolpyruvate carboxykinase 1, cytosolic</td>
<td>Mm01247058_m1</td>
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<tr>
<td>Srebf1</td>
<td>Sterol regulatory element-binding transcription factor 1</td>
<td>Mm00550338_m1</td>
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<tr>
<td>Gapdh</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Mm99999915_g1</td>
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<td>Rn18s</td>
<td>18S ribosomal RNA</td>
<td>Mm03928990_g1</td>
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<td>Hypoxanthine phosphoribosyltransferase</td>
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<td>Actb</td>
<td>Beta actin</td>
<td>Mm00607939_s1</td>
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<td>Rplp0</td>
<td>Ribosomal protein, large, P0</td>
<td>Mm00725448_s1</td>
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</table>

All TaqMan primers and probes are from Life Technologies Australia Pty Ltd, VIC, Australia.