Male obesity is associated with changed spermatozoa Cox4i1 mRNA level and altered seminal vesicle fluid composition in a mouse model

Natalie K. Binder1,2,*, John R. Sheedy1, Natalie J. Hannan2†, and David K. Gardner1,†

1Department of Zoology, University of Melbourne, Parkville 3010, VIC, Australia 2Translational Obstetrics Group, Department of Obstetrics and Gynaecology, Mercy Hospital for Women, University of Melbourne, Heidelberg 3084, VIC, Australia

*Correspondence address. Department of Obstetrics and Gynaecology, University of Melbourne, Heidelberg 3084, VIC, Australia. E-mail: nkbinder@unimelb.edu.au

Submitted on July 21, 2014; resubmitted on February 11, 2015; accepted on February 25, 2015

ABSTRACT: The rate of obesity among men of reproductive age has tripled in the last three decades. Previously, we demonstrated that paternal obesity resulted in impaired preimplantation developmental kinetics, compromised post-compaction metabolism and decreased blastocyst cell number when embryos were generated in vivo. Subsequently, using in vitro fertilization we found embryos of obese males to have altered metabolism before compaction, reduced inner cell mass cell number and retarded fetal development—the difference between these two studies being the method of embryo generation and the presence or absence of seminal plasma, respectively. Here, we hypothesize that both sperm and seminal plasma are affected by obesity, compromising embryogenesis and pregnancy health in a cumulative manner. Epididymal sperm and seminal vesicle fluid were collected from normal and obese C57BL/6 mice. RNA and DNA were extracted from spermatozoa for qPCR and global methylation analysis, respectively. Proteomic (Luminex) and metabolomic (GC–MS) techniques were employed to analyse the composition of seminal vesicle fluid. Nuclear encoded cytochrome c oxidase subunit IV isoform 1 (Cox4i1) of the terminal enzyme in the mitochondrial respiratory chain demonstrated significantly increased RNA levels in the sperm of obese males (P < 0.05). Quantitative seminal plasma analysis identified significant changes in levels of the hormones insulin, leptin and estradiol between normal and obese males (P < 0.05). Further, the metabolite composition of seminal vesicle fluid was significantly affected by obesity. Consequently, this study has determined that obesity affects both sperm and seminal plasma composition. The interaction between sperm and seminal plasma warrants further analysis.

Key words: fructose / high-fat / infertility / semen / sperm

Introduction

The rate of obesity has been growing steadily worldwide, and obesity among reproductive age men has increased as much as 3-fold in the last 30 years (National Center for Health Statistics, 2011). Globally, the number of overweight people now outnumber those undernourished three to two (International Federation of Red Cross and Red Crescent Societies, 2011). Almost 3.5 million adults die each year as a direct result of being overweight or obese (World Health Organisation, 2014), with comorbidities including type 2 diabetes, cardiovascular disease and hypertension. There is growing evidence that paternal obesity significantly affects early embryonic development and subsequent fetal and offspring health despite a weak correlation between obesity and basic semen parameters.

Several studies have investigated the effect of male obesity on sperm motility, morphology, count and concentration with varying results (Jensen et al., 2004; Fejes et al., 2005; Magnusdottir et al., 2005; Kort et al., 2006; Qin et al., 2007; Aggerholm et al., 2008; Hammoud et al., 2008; Pauli et al., 2008; Chavarro et al., 2009; Attaman et al., 2012). Altered spermatozoa physiology including aberrant mitochondrial function and reduced DNA integrity have been associated with obesity (Fariello et al., 2012), however, the negative impact of obesity on male reproductive fitness is yet to reach consensus (for review, Hammoud et al., 2012). A recent meta-analysis found no significant difference in sperm concentration between normal and obese men, however, performing a dichotomized analysis of both sperm count and concentration revealed obesity to be associated with an increased incidence of both oligospermia and azoospermia (Sermonda et al., 2013). Of note,
human cohorts are often confounded by lifestyle factors, which may contribute to inter-study inconsistencies. Similar analysis performed in a rodent model of diet-induced obesity found the sperm of obese mice had decreased motility and reduced fertilization capacity, as well as DNA damage and increased oxidative stress (Bakos et al., 2011b).

Mouse models of diet-induced obesity have also been used to investigate the effect of male obesity on embryo quality. Embryos derived from obese fathers have impaired developmental kinetics, including zygotic arrest and delayed cell cycle progression (Mitchell et al., 2011; Binder et al., 2012a). These embryos also have reduced blastocyst cell number and increased glycolytic rate, indicative of poor quality and reduced implantation potential (Lane and Gardner, 1996). Embryos were generated for these studies by mating normal or obese mice to control females. Consequently, both sperm and seminal plasma could have contributed to any obesity associated abnormalities.

Subsequently, by employing in vitro fertilization (IVF) we were able to delineate the effect of male obesity on fertility in the absence of seminal plasma, as embryos were generated from sperm collected from the epididymis and therefore oocytes had no contact with the seminal plasma. Furthermore, the female reproductive tract was not exposed to seminal plasma, as embryos were generated from sperm collected from the epididymis and seminal plasma could have contributed to any obesity associated abnormalities.

In this study, we hypothesized that male diet-induced obesity negatively affects both spermatozoa and seminal plasma, and that these alterations act in a cumulative manner resulting in compromised embryogenesis and fetal development. As such, the aim of this study was to examine heritable transcriptomic and epigenetic changes in sperm of obese males, and to define changes in the composition of seminal vesicle fluid, the major component of seminal plasma, using proteomic and metabolomic approaches. Better understanding the mechanism underpinning the sub-fertility that is concomitant with male obesity is essential to not only improve reproductive fitness, but also safeguard the health of the next generation.

Materials and Methods

Experimental animals and diets

Twenty-four 6-week-old male C57BL/6 mice (Monash Animal Services, Clayton, Australia) were randomly assigned to one of the two diets for 10 weeks: control diet (4.8% fat, meat free rat and mouse chow), or high fat diet (22% fat, SF00-219; Specialty Feeds, Glen Forrest, Australia) manufactured to emulate a ‘Western-style fast food diet’. Mice on these diets were cultured to emulate a ‘Western-style fast food diet’. Mice on these diets were fed ad libitum. Body weight (BW) was recorded weekly, and peritoneal fat deposits, testes and seminal vesicles measured at euthanasia.

Sample collection

Following the 10-week feeding period, 12 normal and 12 obese male mice were sacrificed for sperm and seminal vesicle fluid collection. Both cauda epididymides from each male were dissected clean of fat and connective tissue and placed into 1 ml of ice-cold isotonic saline in an organ well dish (BD Falcon, NJ, USA). Each cauda epididymis was punctured five times with a 26G needle, and sperm allowed to swim out for 15 min, yielding samples >99% pure. Cauda epididymides were removed from the well, a 20-μl aliquot of sample retained for analysis of basic sperm parameters, and the remainder of each sperm sample snap frozen and stored at −80°C. Samples were randomly allocated to either RNA or DNA extraction for subsequent analysis (six normal and six obese samples for each). Seminal vesicle fluid was collected concurrently. Both seminal vesicles from each male were collected into 1 ml of ice-cold isotonic saline in an organ well dish. A modified glass Pasteur pipette, flame-polished to create a smooth hooked end, was used to gently squeeze the fluid from each seminal vesicle. Each sample was divided into two aliquots, snap frozen and stored at −80°C for subsequent proteomic and metabolomic analysis (12 normal and 12 obese samples for each).

Sperm count, motility and acrosomal loss

A Makler counting chamber was used to determine total sperm count in millions per milliliter. The motility of spermatozoa in room temperature isotonic saline was determined; spermatozoa were considered motile if progression or regular flagellum movement was observed. Sperm acrosomal loss was determined using a similar method to Pope et al. (1991), where 10 μl of sperm in isotonic saline was probed 1:1 with a stain of 1% (w/v) rose bengal, 1% (w/v) fast green FCF and 40% (v/v) ethanol in citric acid (0.1 mol/l) di-sodium phosphate (0.2 mol/l) buffer (pH 7.2–7.3) (unless otherwise stated, all chemicals were obtained from Sigma-Aldrich, St. Louis, MO, USA). Ten microliters of this mixture were smeared onto a glass slide, air-dried at 37°C, and examined with bright field microscopy under oil emersion at ×1000 magnification. The intact acrosome stained purple-blue and the sperm head stained pink. If acrosomal loss had occurred, only pink staining was visible. At least 150 spermatozoa were observed per sample for acrosomal loss.

qPCR array

Total RNA was extracted from ~20 million spermatozoa per sample using the High Pure RNA Isolation Kit (Roche, Penzberg, Germany). Snap frozen samples were thawed on ice, centrifuged at 2000g for 10 min, supernatants removed and pellets resuspended in 200 μl phosphate buffered saline (PBS) + 400 μl lysis buffer. Samples were incubated on ice for 10 min before being homogenized by repeated aspiration and expulsion through a 26G needle. Lysates were transferred to spin filter columns containing glass filter frit that binds nucleic acid (but not contaminating substances). DNase I was added to the column to digest DNA directly on the filter frit and the column was centrifuged at 8000g for 15 s and washed with wash buffer (supplied by the manufacturer) to remove digested DNA and contaminants. RNA was then eluted with a low-salt buffer. RNA concentrations ranging between 22.1 and 49.8 ng/μl were quantified using a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA). Two hundred and twenty nanograms of RNA per sample were converted to cDNA using the Roche Transcriptor High Fidelity cDNA Synthesis Kit. The Roche RealTime ready Configurator (http://www.realtime-ready.roche.com) was used to generate Custom RealTime ready Panels in 384-well format, containing function tested, pre-plated qPCR assays for 24 genes of interest, 3 housekeeping genes and 5 quality controls (four biological samples in triplicate per plate). The quality control assays include three positive controls to test for degradation of initial RNA and
quality of cDNA synthesis, and two minus controls to control for any potential residual genomic DNA. The genes of interest were selected based on previous findings related to the effects of paternal obesity on embryo, fetal and placental development (Binder et al., 2012a, b) (Table I). Each assay well contained a gene-specific primer and a Universal ProbeLibrary Probe (short FAM-labelled hydrolysis probe containing locked nucleic acid). Eight microliters of master mix (LightCycler 480 Probes Master; Roche) and 2 μl of 1:20 diluted cDNA was added to each assay well, yielding a cDNA concentration of 2 ng equivalent total RNA per PCR reaction (assay sensitivity: 50 pg to 50 ng equivalent total RNA per PCR reaction). Quantification of gene expression levels by qPCR was carried out on the LightCycler 480 Instrument (Roche), with the run conditions: 95°C for 10 min; 95°C for 10 s, 60°C for 30 s and 72°C for 1 s (45 cycles; with 40 cycles chosen as the cut-off point); 40°C for 30 s. The technical plate-to-plate variance using RealTime ready assays in conjunction with the LightCycler 480 Probes Master and the LightCycler 480 Instrument is negligible; inter-plate correction with a universal calibrator was not necessary. Relative quantification was determined using the comparative CT method.

**Global methylation ELISA**

DNA was extracted from sperm using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Snap frozen samples were thawed on ice and 300 μl aliquots of ~7 × 10⁸ spermatozoa were centrifuged at 300 g for 5 min, supernatants removed and pellets resuspended in 200 μl PBS with 10% (v/v) protease K. Two hundred microliters of lysis buffer were added to samples and vortexed. Samples were incubated at 56°C for 10 min, followed by addition of 200 μl of ethanol and vortexed. Lysates were transferred to spin filters columns followed by several brief wash and spin cycles to remove ethanol and other contaminants. Columns were then spun dry and the DNA eluted in an elution buffer. Concentration of eluted DNA was determined using a Nanodrop ND 1000 spectrophotometer. Global methylation status was quantified using a 5-methylcytosine ELISA-like assay (MethylFlash Methylated DNA Quantification Kit—Colorimetric, Epigentek, Farmingdale, NY, USA). A negative control (10.0 ng unmethylated polynucleotide containing 50% cytosine), a five-point standard curve (0.5, 1.0, 2.0, 3.0 and 10.0 ng of methylated polynucleotide containing 50% 5-methylcytosine), and 50 ng of DNA per sample (in duplicate) were pipetted into a 96-well plate (treated to have high DNA affinity) and incubated at 37°C for 90 min. The plate was then washed and capture antibody was applied for 60 min at room temperature. Capture antibody was washed away and detection antibody added for 30 min at room temperature. Detection antibody was washed away and signal enhancer applied for 30 min at room temperature. Wells were then washed and the signal was developed. The plate was monitored away from light for up to 10 min until developed (both the standard curve wells and sample wells). Stop solution was added and the plate shaken for 1 min before absorbance was read at 450 nm on a microplate reader (xMark, Bio-Rad, Hercules, CA, USA). Absolute quantification of the amount of methylated DNA in each sample was determined from the standard curve.

**Table I Genes assessed using pathway focused RT-qPCR arrays.**

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paternally expressed—imprinted</td>
<td>Peg10</td>
<td>Paternally expressed 10</td>
</tr>
<tr>
<td></td>
<td>Peg3</td>
<td>Paternally expressed 3</td>
</tr>
<tr>
<td></td>
<td>Nest</td>
<td>Mesoderm-specific transcript</td>
</tr>
<tr>
<td></td>
<td>Igf2</td>
<td>Insulin-like growth factor 2</td>
</tr>
<tr>
<td>Metabolic</td>
<td>Pkd4</td>
<td>Pyruvate dehydrogenase kinase</td>
</tr>
<tr>
<td></td>
<td>Ppara</td>
<td>Peroxisome proliferator-activated receptor alpha</td>
</tr>
<tr>
<td>Cytokines and growth factors</td>
<td>Trf</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td></td>
<td>Il2</td>
<td>Interleukin 2 receptor, alpha</td>
</tr>
<tr>
<td></td>
<td>Prok1</td>
<td>Prokinetin 1</td>
</tr>
<tr>
<td></td>
<td>Cxcr4</td>
<td>Chemokine (C-X-C motif) receptor 4</td>
</tr>
<tr>
<td></td>
<td>Egfr</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>Trophoderm and peri-implantation</td>
<td>Mmp2</td>
<td>Matrix metalloproteinase 2</td>
</tr>
<tr>
<td></td>
<td>Lf</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>Oxidative and ER stress</td>
<td>Casp1</td>
<td>Caspase 12</td>
</tr>
<tr>
<td></td>
<td>Sod1</td>
<td>Superoxide dismutase, soluble</td>
</tr>
<tr>
<td>Signaling, receptors, transcription and translation</td>
<td>Bmpr2</td>
<td>Bone morphogenic protein receptor, type II</td>
</tr>
<tr>
<td></td>
<td>Ctnnb1</td>
<td>Catenin (cadherin associated protein), beta 1</td>
</tr>
<tr>
<td></td>
<td>Stat3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td></td>
<td>Ntr3c1</td>
<td>Nuclear receptor subfamily 3, group C, member 1</td>
</tr>
<tr>
<td>Spermatogenesis</td>
<td>Kdm3a</td>
<td>Lysine (K)-specific demethylase 3A</td>
</tr>
<tr>
<td></td>
<td>Tnp1</td>
<td>Transition protein 1</td>
</tr>
<tr>
<td></td>
<td>Prm1</td>
<td>Protamine 1</td>
</tr>
<tr>
<td>Mitochondria (nuclear encoded)</td>
<td>Cox4l</td>
<td>Cytochrome c oxidase subunit IV isoform 1</td>
</tr>
<tr>
<td></td>
<td>Nrf1</td>
<td>Nuclear respiratory factor 1</td>
</tr>
<tr>
<td>Housekeeping</td>
<td>Hprt1</td>
<td>Hypoxanthine guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td></td>
<td>Tbp</td>
<td>TATA box binding protein</td>
</tr>
<tr>
<td></td>
<td>B2m</td>
<td>Beta-2 microglobulin</td>
</tr>
</tbody>
</table>
Luminex proteomic analysis

Seminal vesicle fluid samples were thawed on ice and homogenized. Homogenates were centrifuged at 2000g for 10 min and the supernatants transferred to fresh 1.5 ml tubes. Total protein was quantified in each sample using a colorimetric bicinchoninic acid protein assay (Pierce BCA Protein Assay Kit, Thermo Scientific, Waltham, MA, USA) to ensure sufficient protein for analysis. Luminex xMAP platform, Milliplex MAP kits were used to simultaneously quantify seminal vesicle fluid concentrations of interleukin (IL)-6, insulin, leptin, monocyte chemotactic protein 1 (CCL2), plasminogen activator inhibitor-1 (PAI-1) total, resistin, tumor necrosis factor-α (TNFα) (using Mouse Serum Adipokine Kit, EMD Millipore, Billerica, MA, USA), and cortisol, estradiol, progesterone, triiodothyronine (T3), thyroxine (T4) (steroid-thyroid hormone—magnetic, EMD Millipore). Neat samples were assayed in duplicate. Plate wells were pre-wet with 200 μl assay buffer for 10 min and then aspirated using a vacuum manifold or decanted (magnetic kit). Standards and samples (25 μl) were added to appropriate wells, followed by addition of assay beads. Plates were incubated overnight (16–18 h) with mild agitation at 4°C, then fluid removed and wells washed twice with wash buffer. Detection antibodies were added to each well and incubated for 1 h at room temperature. The fluorescent conjugate streptavidin—phycoerythrin was then added to each well and plates incubated for 30 min at room temperature. Fluid was then removed and wells washed twice. The intra-assay variability was <10%. Data were collected and analyzed using a BioPlex 200 instrument equipped with BioManager analysis software (Bio-Rad).

GC–MS metabolomic analysis

Pooled biological quality controls (PBQCs) were prepared by combining 40 μl of each previously aliquoted and saline-diluted seminal vesicle fluid sample (n = 12 normal and 12 obese). Individual 20 μl aliquots of seminal vesicle fluid and PBQC were mixed 1 : 1 with chilled chloroform and vortexed for 10 s. Sixty microliters of chilled methanol containing internal standards (1.12 ml methanol, 40 μl 13C-sorbitol [1 mmol/l], 40 μl 13C15N-valine [10 mmol/l]) was added and vortexed. Samples were centrifuged at 16 100g for 5 min at 0°C and the supernatants transferred to fresh 1.5 ml tubes, and diluted with 40 μl distilled, filtered water. Samples were then vortexed and centrifuged at 16 100g for 5 min at 0°C, and 60 μl of the upper polar aqueous phase transferred to glass inserts (Agilent Technologies, Mulgrave, Australia). Samples were dried by speed vacuum at 35°C, then 30 μl of methanol added, and samples dried again. Twenty microliters of methoxamine reagent were added and samples incubated for 2 h on a shaker at 37°C. Twenty microliters of trimethylsilyl derivatizing agent were added and samples incubated for a further 1 h before being rested at room temperature for 1 h. An Agilent 7890A GC and EI/Cl 5975C mass spectrometer was used to collect GC–MS total ion chromatograms (TICs) of the polar extract containing carbohydrates present in the seminal vesicle fluid, using a 7°C min⁻¹ oven ramp. GC Chemstation Instrument Analysis and Gerstel Maestro software packages were used to operate the instrument and export acquired data. The GC–MS experimental parameters, hardware and column used in this experiment have been described elsewhere (De Souza, 2013).

GC–MS data processing and analysis

The TICs were integrated using Chemstation MSD Enhanced Data Analysis software (Agilent) and data with average retention times (RTAVG) between 6.13 and 31.71 min retained for processing. A Python-based automated retention time correction/dynamic programing algorithm was used to align the TICs from each sample using previously reported methodology (Robinson et al., 2007; O’Callaghan et al., 2012). Two samples from the normal group and two from the obese group were removed from further analysis due to peak overlapping. A data matrix of aligned and integrated GC–MS TIC signals (n = 10 normal and 10 obese) was imported into the R software package where scedasticity was assessed and data logarithm transformed and normalized by the median integral area of the data matrix, as previously reported (Gook et al., 2014; R Core Team, 2014). The data matrix then underwent principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) (The Unscrambler software, CAMO, Oslo, Norway) after centering and scaling. The first two components explained 89% of covariance by PCA and 87% by PLS-DA. As described previously (Sheedy et al., 2014), this method allows data reduction and pattern recognition analysis, which provided a list of 43 peaks from the 153 peaks extracted from the GC–MS TICs, which discriminated between seminal vesicle fluid samples from the normal and obese groups. Wilcoxon signed-rank tests were performed for the 43 peaks comparing normal and obese groups in R (R Core Team, 2014), which were filtered down to nine peaks of interest, where P < 0.05. Chemstation MSD software and the NIST MS Search Program version 2.0 was used to identify the peaks. No correction for multiple testing was performed.

Statistical analysis

After testing the distribution of the data with the D’Agostino-Pearson omnibus normality test, statistical analysis was performed on raw data. Mouse and tissue weight were normally distributed and statistically analyzed with a parametric t-test. Sperm motility was normally distributed and statistically analyzed with a parametric t-test. Sperm acrosomal loss was normally distributed and statistically analyzed with a parametric t-test. Sperm count was not normally distributed and statistically analyzed non-parametrically using the Mann–Whitney test. Global DNA methylation was not normally distributed and statistically analyzed non-parametrically using the Mann–Whitney test. Multiplex proteomic analysis was not normally distributed and statistically analyzed non-parametrically using the Mann–Whitney test. For qPCR array data, each gene detectable below the limit of detection (cycle 40) was analyzed individually, without assuming a consistent standard deviation. Statistical analysis was done using PRISM version 6.00 for Windows (GraphPad, San Diego, CA, USA). GC–MS data were assessed as detailed above. P-values <0.05 were considered statistically significant. No correction for multiple testing was performed.

Animal ethics

This study was carried out in strict accordance with the Australian code of practice for the care and use of animals for scientific purposes. Ethical approval was obtained from The University of Melbourne Animal Ethics Committee (ID#1011628.1) prior to experimentation.

Results

Dietary effects on weight gain and fat deposits

Male C57BL/6 mice fed a high fat diet for 10 weeks (obese) gained significantly more weight than mice fed a control diet (normal). Importantly, the obese mice were significantly heavier at the time of sample collection than the normal mice. Obese mice also had significantly increased peritoneal fat deposits compared with normal mice, which did not normalize with weight (Table I).

Obesity effects on basic sperm parameters

Sperm collected from normal and obese male mice did not differ in basic parameters of count, motility and acrosomal loss (Fig. 1).
Obesity effects on spermatozoa RNA and global DNA methylation

Of the 24 genes investigated, Peg10, Lif, Ctnnb1, Tnp1, Prm1 and Cox4i1 had detectable levels of mRNA present in sperm (Supplementary data, Table SI). Significantly more Cox4i1 mRNA was present in the sperm from obese males compared with sperm from normal males (Fig. 2, \( P = 0.0205 \)). Of the three housekeeping genes selected for this array, only B2m was present at consistent levels in all sperm samples. As such, the other two housekeeping genes Hprt1 and Tbp were not used for the analysis. Global methylation of DNA extracted from spermatozoa did not differ significantly between normal and obese male mice (1.0 \( \pm \) 0.2 versus 1.1 \( \pm \) 0.3, respectively).

Obesity effects on seminal vesicle fluid composition

Of the 12 analytes investigated, IL-6, insulin, leptin, CCL2, PAI-1 total, resistin, TNF\( \alpha \), cortisol, estradiol and T3 were present in seminal vesicle fluid. Interestingly, in mouse seminal vesicle fluid, both progesterone and T4 were below the limit of detection of the assay (Table III). Seminal vesicle fluid collected from obese mice had significantly increased levels of insulin (\( P = 0.0488 \)) and leptin (\( P = 0.0331 \)), and significantly decreased levels of estradiol (\( P = 0.0373 \)) compared with normal (Fig. 3). PCA and PLS-DA analysis of the GC–MS profiles showed differences between seminal vesicle fluid samples from either normal or obese mice (Fig. 4). Metabolites identified as significantly contributing (\( P < 0.05 \)) to the difference between normal and obese groups were myo-inositol, fructose, glycerol phosphate, glycine, isoleucine, glutamic acid, unmethoxymated hexose, threonine and taurine (Table IV).

Discussion

While a negative effect of male obesity on reproductive health is now more widely recognized, the underlying mechanisms resulting in diminished embryo quality and compromised fetal health are yet to be fully elucidated. Furthermore, the extent of the effect, if any, of obesity on semen parameters remains inconclusive. The data from this study demonstrate for the first time that obesity is associated with changes in both spermatozoa RNA content and seminal vesicle fluid constitution. Despite obesity having no effect on basic sperm parameters in this model, the observed aberrations at the metabolomic, proteomic and transcriptomic levels may act in a cumulative manner, resulting in subfertility concomitant with obesity.

Total sperm count, sperm motility and the proportion of acrosomal loss did not differ significantly with obesity. Numerous studies have looked at correlating basic sperm parameters with obesity (Jensen et al., 2004; Fejes et al., 2005; Magnusdottir et al., 2005; Kort et al., 2010).
In humans, often interpretation of results is complicated by confounding lifestyle factors and variations in collection of samples and analysis, therefore, results thus far have been inconclusive. Several rodent studies have reported decreased sperm motility with obesity (Ghanayem et al., 2010; Fernandez et al., 2011; Bakos et al., 2011b; Palmer et al., 2012), and one study also reported no effect of obesity on sperm motility (Palmer et al., 2012). All of these studies found no effect of obesity on sperm count, and only one found decreased sperm morphology with obesity (Palmer et al., 2012). The sperm motility assay carried out in this study used basic cell preparation and likely induced cold shock in the spermatozoa as a result of rapid cooling. Potentially, a protocol more similar to those used for IVF and maintaining physiological temperature would yield different motility scores. However, it is also important to note that parameters such as gross motility do not necessarily correlate with molecular aberrations of the spermatozoa, nor can sperm count readily reflect physiology. To understand the effect of obesity on fertility better, a more in depth analysis of seminal fluid is necessary.

**Table III**  Analytes assessed in mouse seminal vesicle fluid using Luminex multiplex analysis.

<table>
<thead>
<tr>
<th>Milliplex</th>
<th>Analyte detected</th>
<th>Sample range, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse serum adipokine</td>
<td>IL-6  Yes</td>
<td>1.4–11.8</td>
</tr>
<tr>
<td>Mouse serum adipokine</td>
<td>Insulin Yes</td>
<td>58–380</td>
</tr>
<tr>
<td>Mouse serum adipokine</td>
<td>Leptin Yes</td>
<td>32–310</td>
</tr>
<tr>
<td>Steroid-thyroid hormone</td>
<td>CCL2 Yes</td>
<td>1–43</td>
</tr>
<tr>
<td>Steroid-thyroid hormone</td>
<td>PAI-1 (total) Yes</td>
<td>10–1600</td>
</tr>
<tr>
<td>Steroid-thyroid hormone</td>
<td>Resistin Yes</td>
<td>31–1126</td>
</tr>
<tr>
<td>Steroid-thyroid hormone</td>
<td>TNFα Yes</td>
<td>0.9–4.6</td>
</tr>
<tr>
<td>Steroid-thyroid hormone</td>
<td>Cortisol Yes</td>
<td>7820–2920</td>
</tr>
<tr>
<td>Steroid-thyroid hormone</td>
<td>Estradiol Yes</td>
<td>1230–3570</td>
</tr>
<tr>
<td>Steroid-thyroid hormone</td>
<td>Progesterone No</td>
<td>N/A</td>
</tr>
<tr>
<td>Steroid-thyroid hormone</td>
<td>T3 Yes</td>
<td>160–1070</td>
</tr>
<tr>
<td>Steroid-thyroid hormone</td>
<td>T4 No</td>
<td>N/A</td>
</tr>
</tbody>
</table>

IL-6, interleukin 6; CCL2, monocyte chemotactic protein 1; PAI-1, plasminogen activator inhibitor-1; TNFα, tumor necrosis factor-α; T3, triiodothyronine; T4, thyroxin.

Lower detectable concentration plus two standard deviations for progesterone and T4 are 90 and 280 pg/ml, respectively (as supplied by the manufacturer).
In this study, transcriptome analysis identified significantly higher levels of cytochrome c oxidase subunit IV isoform 1 (Cox4i1) mRNA in sperm from obese males compared with normal. Nuclear encoded Cox4i1 is part of the terminal enzyme of the mitochondrial respiratory electron transport chain and the protein localizes to the inner mitochondrial membrane (Blake et al., 2014). Deletion of Cox4 in the yeast Saccharomyces cerevisiae affects mitochondrial membrane potential (Miceli et al., 2012). We have previously shown that 4-cell embryos generated from the sperm of obese males have significantly decreased mitochondrial membrane potential and increased pyruvate metabolism (Binder et al., 2012a). It is likely that mRNA present in the spermatozoa has a functional role in early embryonic development (Hosken and Hodgson, 2014).

Until recently, it was believed that the male contribution to the developing embryo was limited to a haploid genome, centriole (in most species) and calcium oscillation induction (Schatten, 1994; Bornens, 2012). Emerging research has since shown that 4-cell embryos generated from the sperm of obese males have significantly decreased mitochondrial membrane potential and increased pyruvate metabolism (Binder et al., 2012a). It is likely that mRNA present in the spermatozoa has a functional role in early embryonic development (Hosken and Hodgson, 2014).

Table IV Metabolites identified as significantly contributing ($P < 0.05$) to the difference between seminal vesicle fluid from normal and obese groups.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Obesity effect</th>
<th>$\text{RT}_{\text{AVG}}$ (min)</th>
<th>Quantified ion (m/z)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo-inositol</td>
<td>Decreased</td>
<td>23.73</td>
<td>318.00</td>
<td>0.0015</td>
</tr>
<tr>
<td>Fructose</td>
<td>Increased</td>
<td>20.09</td>
<td>214.00</td>
<td>0.0039</td>
</tr>
<tr>
<td>Glycerol phosphate</td>
<td>Decreased</td>
<td>18.54</td>
<td>243.00</td>
<td>0.0068</td>
</tr>
<tr>
<td>Glycine</td>
<td>Decreased</td>
<td>11.33</td>
<td>248.00</td>
<td>0.0089</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Decreased</td>
<td>11.08</td>
<td>218.00</td>
<td>0.0147</td>
</tr>
<tr>
<td>Glumatic acid</td>
<td>Decreased</td>
<td>16.89</td>
<td>246.00</td>
<td>0.0232</td>
</tr>
<tr>
<td>Unmethoximated hexose</td>
<td>Decreased</td>
<td>21.01</td>
<td>204.00</td>
<td>0.0288</td>
</tr>
<tr>
<td>Threonine</td>
<td>Decreased</td>
<td>12.78</td>
<td>291.00</td>
<td>0.0355</td>
</tr>
<tr>
<td>Taurine</td>
<td>Increased</td>
<td>17.72</td>
<td>328.00</td>
<td>0.0433</td>
</tr>
</tbody>
</table>

$\text{RT}_{\text{AVG}}$, average retention time.

Figure 4 The carbohydrate composition of seminal vesicle fluid from normal and obese mice was qualitatively different. PCA (A) and partial least squares discriminant analysis (B) two-dimensional score plots comparing seminal vesicle fluid composition by GC–MS from normal (dark) and obese (light) male mice. Each score represents the sum of the integrated GC–MS TICs. $n = 10$ males per diet.

In this study, transcriptome analysis identified significantly higher levels of cytochrome c oxidase subunit IV isoform 1 (Cox4i1) mRNA in sperm from obese males compared with normal. Nuclear encoded Cox4i1 is part of the terminal enzyme of the mitochondrial respiratory electron transport chain and the protein localizes to the inner mitochondrial membrane (Blake et al., 2014). Deletion of Cox4 in the yeast Saccharomyces cerevisiae affects mitochondrial membrane potential (Miceli et al., 2012). We have previously shown that 4-cell embryos generated from the sperm of obese males have significantly decreased mitochondrial membrane potential and increased pyruvate metabolism (Binder et al., 2012a). It is likely that mRNA present in the spermatozoa has a functional role in early embryonic development (Hosken and Hodgson, 2014).

Until recently, it was believed that the male contribution to the developing embryo was limited to a haploid genome, centriole (in most species) and calcium oscillation induction (Schatten, 1994; Bornens, 2012). Emerging research has since shown that a paternal load of RNA is delivered into the oocyte by the fertilizing sperm, and this RNA is translated in the zygote, having a direct role in early embryogenesis. Work in model organism Caenorhabditis elegans has identified hundreds of paternally derived mRNA present in the 1-cell embryo, prior to initiation of transcription (Stoeckius et al., 2014). Furthermore, evidence suggests that this RNA load, the majority of which is related to growth, is selectively transferred and not just present as random spill-over from spermogenesis. Spermatozoa RNA may be a transgenerational carrier of environmental information (Gapp et al., 2014).

Given this change in spermatozoa mRNA load, epigenetic analysis was undertaken. Global methylation of spermatozoa DNA was unaffected by paternal obesity. The methylation assay used in this study quantifies the total amount of 5-methylcytosine in a given DNA sample and does not look at CpG island specific methylation (gene-specific changes). While there is no gross change in DNA methylation between the normal and obese groups, individual genes may vary in methylation status (beyond the detection limit of this assay) and thus contribute to heritable epigenetic alterations with paternal obesity. Further investigation of gene and promoter-specific methylation changes is required.

Fluid from the seminal vesicle forms the major component of seminal plasma. Proteins of seminal vesicle fluid undergo considerable turnover, indicating a capacity to rapidly modulate fluid composition in response to environmental and evolutionary stimuli (Claydon et al., 2012). Seminal plasma...
proteins are known to affect the functionality of spermatozoa in several ways including motility, membrane fluidity and fertilization ability (Mansskova et al., 2003; Caballero et al., 2004; Rodriguez-Martinez et al., 2011; Piel et al., 2013). Seminal vesicle fluid from obese males differs significantly from normal in both protein and metabolite composition. Recently, analysis of human seminal plasma has identified increased levels of both leptin and insulin in obese men (Leisegang et al., 2014), consistent with the findings of this study. Both leptin and insulin receptors are present on the plasma membrane of mammalian spermatozoa (Jope et al., 2003; De Ambrogi et al., 2007; Aquila et al., 2008; Carpino et al., 2010). Given that leptin and insulin are involved in endocrine signaling with spermatozoa (Aquila et al., 2005a, b), it is possible that during ejaculation and transit through the female reproductive tract, the altered levels of these hormones with obesity will affect sperm function.

Estradiol, produced from the aromatization of testosterone (Naftolin et al., 1975), is significantly reduced in the seminal vesicle fluid of obese mice. Decreased circulating testosterone has been reported in this animal model of diet-induced obesity (Bakos et al., 2011b; Palmer et al., 2012) as well as obese men (Gutorova et al., 2014; Vandewalle et al., 2014; Zhao et al., 2014), and is likely due to the lipid-soluble androgen preferentially accumulating in adipose tissue (Aziz, 1989; Gambineri et al., 2002). Adipose tissue is an active endocrine organ and readily aromatizes testosterone to estradiol (Kley et al., 1980) resulting in significantly increased circulating estradiol levels in obese men (Hammoud et al., 2009; Tunc et al., 2011; Luconi et al., 2013; Samavat et al., 2014). This discrepancy in levels of circulating estradiol and seminal vesicle estradiol suggests that much like the other glands and tissues of the male reproductive system, a blood-tissue barrier may result in this tissue-specific effect (Mital et al., 2011; Cheng and Mruk, 2012).

Fructose is the major glycolysable substrate of seminal plasma and is significantly increased in the seminal vesicle fluid of obese male mice, similar to obese human males (Martini et al., 2010). There have been a number of conflicting reports regarding the up- and down-regulation of seminal fructose with male infertility (Lewis-Jones et al., 1996; Andrade-Rocha, 1999; Al-Daghistani et al., 2010). Richthoff and colleagues found seminal fructose correlated positively with sperm DNA fragmentation (Richthoff et al., 2002), and different etiologies of male infertility have been linked to either increased or decreased seminal fructose levels (Said et al., 2009; Jayaraman et al., 2014). Importantly, fructose is widely accepted as a marker of seminal vesicle function (Ndovi et al., 2006; Raj et al., 2014).

Taurine, widely recognized for its antioxidant capabilities, is also significantly increased in the seminal vesicle fluid of obese mice. Mammalian spermatozoa are highly susceptible to oxidative damage (Aitken and Curry, 2011), and increased intracellular reactive oxygen species (ROS) have been identified with male obesity (Bakos et al., 2011b). In fact, it is the taurine precursor hypotaurine that acts as an antioxidant in spermatozoa and is oxidized to taurine in the process (Holmes et al., 1992). As taurine is the by-product of free radical scavenging, it is possible that the increased taurine identified in seminal vesicle fluid of obese males is due to antioxidant action in response to elevated ROS levels.

Furthermore, seminal plasma is more than just a transport medium for spermatozoa; it also acts directly on the female reproductive tract, potentially influencing embryonic development and implantation. In Drosophila melanogaster, components of the seminal fluid have been found to target the uterus, oviduct, ovary and oocyte, acting to induce physiological changes (Ravi Ram et al., 2005). Also, seminal plasma has been shown to instigate changes in the cytokine environment of the uterus that are necessary for preimplantation embryo development and subsequent attachment and implantation (Tremellen et al., 1998). Previously we have demonstrated that local signaling mediators can significantly alter the endometrium and embryo (Hannan et al., 2011; Paiva et al., 2011), perhaps not surprising given the sensitivity of the early embryo (Gardner and Lane, 2005; Lane and Gardner, 2005). Therefore, alterations in the local uterine microenvironment, in response to suboptimal seminal plasma (associated with male obesity), may have consequences on implantation and also offspring health.

Importantly, no correction for multiple testing was performed. Multiple testing could result in false positives and as such these results need to be further replicated. For each data set involving multiple testing (RNA levels, protein concentrations and metabolite profiles), the number of significant results was greater than the false-positive rate due to type 1 error). Further analysis with a larger cohort is likely to reveal a number of additional significant changes to the germine and seminal plasma brought about by male obesity.

In conclusion, this is the first study to combined metabolomic, proteomic and transcriptomic analysis of either spermatozoa or seminal vesicle fluid from obese males. Despite sperm count, motility and acrosomal loss being comparable between normal and obese males, this study clearly demonstrates that both mRNA levels in the spermatozoa and the protein and metabolite milieu in seminal vesicle fluid are significantly affected by obesity. Spermatozoa mRNA plays a role in early embryonic development, and seminal plasma interactions with both gametes and the female reproductive tract are important for the establishment of a healthy pregnancy. Studies expanding on the transcriptomic analysis of spermatozoa will likely yield significant insight into the mechanism underpinning the negative effect of paternal obesity on embryo development and offspring health.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

**Acknowledgements**

We thank Drs Brunda Nijjagal, David De Souza and Sean O’Callaghan from Metabolomics Australia (Bio21 Institute, The University of Melbourne) for their contribution to the GC–MS sample preparation methodology, data collection and data processing and Dr Louise Zieseniss for technical support and assistance.

**Authors’ roles**

N.K.B., N.J.H. and D.K.G. designed the study. N.K.B. generated the samples and performed the experiments and data analysis, except for the GC–MS which was performed by Metabolomics Australia and interpreted and analyzed by J.R.S. All authors were involved in critical discussion of the data and manuscript drafting and review.

**Funding**

This work was supported by the University of Melbourne [to D.K.G.]; the National Health and Medical Research Council [Early Career...
Fellowship #628927 to N.J.H.; the Australian Postgraduate Award [to N.K.B.]; the Norma Hilda Schuster (nee Swift) Scholarship [to N.K.B.] and the Jasper Loftus-Hills Memorial Award [to N.K.B.].

Conflict of interest
The authors have no conflicting interests to declare.

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