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Vertical sleeve gastrectomy improves indices of metabolic disease in rodent model of surgical menopause

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Abstract

Objective—Though females are the most common recipients of weight loss surgeries for the amelioration of the comorbidities of obesity, few studies have addressed the efficacy of these procedures with specific attention to reproductive stage. Here we ask in a rodent model of vertical sleeve gastrectomy (VSG) whether improvements to metabolic health are realized in females having received surgical menopause. Specifically we were interested in knowing whether rats made menopausal through surgical means would exhibit persistent hepatic steatosis as reported in previously pregnant, freely-cycling female VSG rats or if it is resolved as reported in male VSG rats.

Methods—All the rats first received ovariectomy (OVX) and then were placed on high fat diet (HFD) prior to either sham or VSG surgery (N = 12, 9) and then were monitored for resolution of obesity-related comorbidities.

Results—VSG was sufficient to reduce weight and adiposity in OVX females in comparison to Obese rats ($P < 0.001$). Glucose tolerance ($P < 0.05$) was improved in OVX-VSG females with no change in insulin sensitivity. Both circulating ($P < 0.01$) and hepatic triglyceride ($P < 0.01$) levels were also reduced after VSG. Liver integrity was improved in OVX-VSG in comparison to OVX-Obese as reflected by reduced aspartate aminotransferase (AST) levels ($P < 0.05$). The ability of mitochondria to generate ATP was maintained and an increase in complex IV may decrease the production of mitochondrial ROS.

Conclusions—Taken together, VSG in ovariectomized animals experience many positive benefits including the resolution of hepatic steatosis that persists in reproductively-intact female rats after VSG.

Keywords

vertical sleeve gastrectomy; bariatric surgery; menopause; obesity; mitochondria; hepatic

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INTRODUCTION

Women aged 40-59 years of age (y) have the highest prevalence of obesity in comparison to either males of the same age or females of other age categories [1]. This age bracket encompasses women who are naturally transitioning to menopause which begins between 45-55 y and is completed by around 60 y. Menopause is the permanent cessation of menstrual periods that occurs with ageing. Menopause can also be induced by surgical removal of the ovaries and it may occur as a side effect of chemotherapy or radiation treatments. Menopause results in part from a reduction in both estrogen and progesterone production by the ovaries. Because many body tissues are estrogen-responsive, the perimenopausal sequelae are substantial and diverse, but a common effect is weight gain. The perimenopausal side effects include vasomotor symptoms such as sweats and hot flashes, vaginal dryness, sexual dysfunction, urinary incontinence, sleep disturbances, memory loss, depression, headaches, fatigue, joint pain and weight gain. With the loss of a protective effect of estrogen on diverse body systems, postmenopausal females are at increased risk for various components of Metabolic Syndrome (MetS) such as heart disease, obesity, diabetes and dyslipidemia.

Bariatric surgery for long term body weight loss and resolution of the comorbidities of obesity has gained popularity in last decades [2, 3]. The vast majority of recipients of bariatric surgeries are women (>80%) [4] of whom approximately half are of the peri/postmenopausal age [5]. Vertical sleeve gastrectomy (VSG) resects 80% of the stomach, creating a tube linking the esophagus to the duodenum. Because of the effectiveness and relative simplicity of VSG surgery in comparison to the gold standard surgery, Roux-en-Y gastric bypass (RYGB), which is riddled with a variety of potential complications, VSG is now on the rise in the U.S. [6, 7].

Many clinical indices show improvement with VSG that rivals the outcomes of RYGB. For example, the reported loss of 60-65% of excess body weight after VSG [8, 9] is similar to RYGB [8]. The remission of comorbidities, such as type-2 diabetes mellitus (T2DM) and hyperlipidemia, is similar for VSG with respect to RYGB. Death due to myocardial infarction and cardiovascular death are reduced by nearly 50% [10]. Overall risk for cancer mortality is reduced [11].

With respect to non-alcoholic fatty liver disease (NAFLD), no studies to date have reported liver biopsies from patients having received VSG comparing peri-operative and post-surgical hepatic histology and steatosis. Numerous studies suggest that hepatic steatosis, fibrosis and inflammation are significantly improved following RYGB [12-16]. These findings are not universal, however, since one study showed an improvement in steatosis but no histologic improvement of fibrosis [17], a condition whose long-term damage may cause increased mortality risk [18].

Though numerous clinical improvements resulting from both RYGB and VSG are promising, stratified analyses for the efficacy of each of these clinical parameters by age and

reproductive status are sparse [19]. This is particularly true with respect to menopausal females who have unique metabolic issues.

Rodent models of VSG and RYGB have recapitulated many of the beneficial outcomes to human bariatric patients such as decreased body weight through fat loss, improvements in glucose tolerance and metabolic hormone changes [20-25]. Rodent models have also revealed at least one negative outcome of VSG surgery that is yet to be reported in the human literature. Premiparous female rats exhibited a worsening of hepatic steatosis in post-VSG [26] whereas in male rats showed a reduction of lipids in the liver [6].

Overall, only one report has specifically addressed the impact of bariatric surgery in a model of menopause. RYGB was used in a surgical model of menopause and the interaction of estradiol replacement and surgical weight loss was reported [27]. No work has been reported about the effect of VSG in a menopause model. The goal of this study is to determine whether VSG is sufficient to produce improvements in global metabolic parameters in a surgical model of menopause, in comparison to previously reported indices in males and reproductively intact females. We also sought to determine whether hepatic steatosis would be improved, as in male rats, or persist and worsen, as in post-partum female rats. Female Long-Evans rats were ovariectomized and allowed to recover for 3 weeks on standard chow and then placed on a high-fat diet (HFD) for an additional 3 weeks. At this point the animals received either sham surgery or VSG. Following 5 weeks of surgical recovery while being maintained on HFD, we interrogated measures of body mass, food intake, body composition, glucose tolerance, and circulating hormones. We focused on liver integrity, hepatic gene expression, lipid levels and liver mitochondrial function.

MATERIALS & METHODS

Animals

All procedures for animal use complied with the Guidelines for the Care and Use of Laboratory Animals by the National Institutes of Health and were reviewed and approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee.

Female Long Evans rats (200-225g) (Harlan, Indianapolis, IN) (N=30) were initially multiply housed and maintained in a room on a 12/12-h light/dark cycle at 25 °C and 50-60% humidity with *ad libitum* access to water and standard chow (#8640, Envigo, 3.0 kCal/g; 17% fat, 54% carbohydrate, 29% protein). After 1 week of acclimatization to the vivarium, female rats received a bilateral ovariectomy. Animals remained on standard chow for 3 weeks and then were switched palatable high-fat diet (HFD) (#D03082706, Research Diets, New Brunswick, NJ, 4.54 kCal/g; 40% fat, 46% carbohydrate, 15% protein) for 3 weeks prior to weight loss surgery and for the remainder of the study. Animals were assigned in a counterbalanced fashion by body weight to receive either sham-vertical sleeve gastrectomy (sham-VSG) or VSG surgery. (N=12, 18). The animals therein were assigned as either OVX-Obese or OVX-VSG. Animals were singly housed thereafter and continued on the study for an additional 6 weeks post-surgery. Animals were euthanized by conscious decapitation for tissue harvest, trunk blood and isolation of liver mitochondria. (Final N=12 (Sham), 9 (VSG)). Reasons for exclusion or loss of animals include post-operative

complications of VSG surgery. Surgical success was similar to previous published studies [26, 28].

Surgical Procedures

Bilateral ovariectomy—Animals are given one dose of carprofen (0.10 mL of 5mg/mL) at the time of surgery. Rats were anesthetized using isoflurane. Two small medial abdominal incisions (0.5 inches long) were made to expose the ovaries. Ovarian tubes were tied off with sterile, absorbable suture, and ovaries removed. The muscular wall was closed with sterile sutures and the skin closed using wound clips.

Surgical weight loss pre-operative care—4 days prior to VSG or sham-VSG surgery, body composition was assessed using an EchoMRI analyzer (Houston, TX). Animals were placed in either Sham or VSG groups in a counterbalanced fashion by body weight and also taking into account body fat composition. Animals were fed Osmolite OneCal liquid diet (#00735, Abbott Laboratories, Columbus, Ohio) but no solid-food for 24 h prior to surgery.

VSG—VSG consisted of a midline abdominal laparotomy with exteriorization of the stomach. Ligaments and connective tissue was removed leaving an easily articulated stomach. The lateral 80% of the stomach was excised using an ENDO GIA Ultra Universal stapler (#EGIAUSHORT, Covidien, MA) coupled with an ENDO GIA Auto Suture Universal Articulating Loading Unit, 45 mm – 2.5 mm (#030454, Covidien, MA). A gastric tube in continuity with the esophagus thus remained. This gastric sleeve was then reintegrated into the abdominal cavity and the abdominal wall was closed in layers using 3-0 coated vicryl suture (#J904, Ethicon).

Sham-VSG—An abdominal laparotomy was performed. Light manual pressure was applied with to the exteriorized stomach and then the abdomen was closed in layers as described above.

Post-operative care—Following surgery, all rats received care for 2d, consisting of once-daily subcutaneous injections of 5 mL saline and carprofen (0.10 mL of 5mg/mL dose) and twice-daily subcutaneous injections of Buprenex® (0.10 mL of 0.3mg/mL dose). Animals were maintained on Osmolite (#00735, Abbott Laboratories, Columbus, OH) until food was returned 3 d following surgery.

Body weight, composition and food intake—Body weights were measured weekly. Following the weight loss surgery, animals were weighted daily for the first 7 days and then weekly thereafter. Body lean and fat mass composition was analyzed using Echo Magnetic Resonance Imaging (EchoMedical Systems, Houston, TX) at the start of the study and 3, 6 and 11 wks.

Glucose tolerance testing—Rats were 6 hour-fasted after the onset of the light cycle. Baseline tail vein blood glucose was measured by Accu-Chek Aviva Plus™ glucometer and glucose strips (Roche, Indianapolis, IN). Animals were then dosed with 50% dextrose (1.5 g/kg body weight) intraperitoneally at time t = 0 min and subsequently blood glucose measured at t = 15, 30, 45, 60 and 120 min post-injection. Tail vein blood also was collected

in heparinized tubes for insulin determination at 0 and 15 min. Insulin measurements were made using an Insulin ELISA (#90060, Crystal Chem INC., IL). Glucose and insulin measurements were made in duplicate.

Insulin tolerance test—Rats were 6 h-fasted following the onset of the light cycle. Baseline blood glucose measurements were made ($t=0$) and then animals dosed with Humulin-R (#HI-213, Lilly, IN) (0.5 U/kg body weight) and tail vein measurements of blood glucose were made using the glucometer at $t = 15, 30, 45$ and 60 .

Tissue harvest—At POD animals were euthanized by conscious decapitation starting at 6 h following the onset of the light cycle.

Isolation of intact liver mitochondria—Hepatic mitochondria were isolated using a modified protocol of published methods [29]. Each freshly excised rat liver was washed in 20-30 mL of ice-cold 10 mM HEPES, pH 7.2, 0.3 M sucrose, 0.2 mM EDTA (HSE), the liver was cut into 1-2 gram sections and the fluid was discarded. The liver sections were minced with a razor for five minutes on an ice-cold plastic surface in the presence of 1 mL HSE and then homogenized for 10 strokes using a loose-fitting glass Dounce homogenizer (Sigma) in 5 mL HSE containing 0.1 mM PMSF and 1 mg/mL fatty acid-free BSA (Sigma). The homogenate was split into microcentrifuge tubes and cellular debris was pelleted by centrifugation at $800 \times g$ for 10 min at 4°C and the supernatants containing mitochondria were recovered. Mitochondria were then pelleted by centrifugation at $8500 \times g$ for 10 min at 4°C . The supernatants were discarded, the pellets were gently resuspended using a 1 mL pipettor and pelleted again at $8500 \times g$ for 10 min at 4°C . Pellets were combined and resuspended in a total volume of 0.5 to 1.0 mL of 220 mM mannitol, 70 mM sucrose, 5 mM Mops, pH 7.4. The protein concentration was determined using the BioRad DC Protein assay.

Assay for changes in the content of mitochondria in liver—Using a portion of the total liver homogenate, prepared as above, citrate synthase activity was measured spectrophotometrically using the Citrate Synthase Assay Kit (#CS0720, Sigma) while the protein concentration of the homogenate was determined using the BioRad DC Protein assay (#5000111, Bio-Rad).

Measurements of intact mitochondrial function—Rates of O_2 consumption by intact liver mitochondria were measured using an Oroboros Oxygraph at 25°C . The 2 mL assay mixture contained 50 mM Mops, 5 mM KH_2PO_4 , 100 mM KCl and 1 mM EGTA, pH 7.4, and mitochondria containing 0.1-0.5 mg protein. In separate experiments, either 20 mM glutamate plus 5 mM malate or 40 μM palmitoyl carnitine plus 5 mM malate was added to obtain the rate of State 2 respiration. ADP was added to 1 mM for State 3 respiration, which is proportional to the rate of ATP synthesis. State 4 respiration was determined by inhibiting ATP synthase with 5 μM oligomycin, and the rate of uncoupled respiration was obtained after adding the weak acid uncoupler carbonyl cyanide-ptrifluoromethoxyphenylhydrazone (FCCP) to 1 μM . The respiratory control ratio (RCR) is the rate of State 3 divided by the rate of State 4.

Preparation of open, washed mitochondrial membranes (MM)—MM were prepared by pelleting 0.2-0.4 mL intact mitochondria at $8500 \times g$ for 10 min at 4°C, followed by resuspension in an equal volume of 50 mM Tris, pH 7.4. The suspension was frozen in liquid nitrogen and thawed rapidly for two cycles, centrifuged as above and resuspended in an equal volume of 50 mM Tris, pH 7.4. Centrifugation and resuspension in Tris buffer was repeated. Protein concentrations were determined using the BioRad DC Protein assay.

Measurements of whole chain electron transfer activity.—Rates of electron transfer from $\text{NADH} \rightarrow \text{O}_2$ and $\text{succinate} \rightarrow \text{O}_2$ were measured as O_2 consumption using the Oroboros Oxygraph. Each assay was performed at 25°C in 2 mL of 50 mM Tris pH 7.4, 39.6 mM KCl, 40 μM horse heart cytochrome *c* and MM containing 0.1-0.2 mg protein. Electron transfer from $\text{NADH} \rightarrow \text{O}_2$ was initiated by the addition of NADH to 400 μM and $\text{succinate} \rightarrow \text{O}_2$ by the addition of 20 mM succinate.

Complex I activity was measured at 25°C by monitoring the oxidation of NADH at 340 nm ($\epsilon = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$) using a Hitachi U-3000 spectrophotometer. The assay mixtures contained 50 mM Tris pH 7.4, 39.6 mM KCl, 200 μM CoQ₁, 3 mM KCN, 5 μM antimycin A and 0.1-0.2 mg MM. The reaction was started by the addition of NADH to 100 μM .

Complex IV activity was measured in the Oroboros Oxygraph in 50 mM Tris pH 7.4, 39.6 mM KCl, 3 mM ascorbate, 0.3 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), 40 μM horse heart cytochrome *c*, 5 μM myxothiazol and 5 μM antimycin A. The reaction was initiated by the addition of MM containing 0.1-0.2 mg protein. The slow rate of non-enzymatic reduction of O_2 by ascorbate/TMPD/cytochrome *c* was subtracted from all rates obtained after the addition of MM.

For the measurements of intact mitochondrial function, we examined intact mitochondria from 3 Sham rats and 4 VSG rats (all chosen at random), and we performed each of the assays twice. For the measurements of citrate synthase activity, whole chain electron transfer activity, complex I activity and complex IV activity, we examined open, washed mitochondrial membranes from 8 Sham rats and 5 VSG rats, and we performed each assay twice.

Measurements of analytes—The following analytes were measured according to the manufacturer's specifications: triglycerides (#T7532-120, Fisher Scientific, VA), cholesterol (#T7-120, Fisher Scientific, VA), alanine aminotransferase (ALT) (#A7526-150, Fisher Scientific, VA), aspartate aminotransferase (AST) (#A7561-150, Fisher Scientific, VA), leptin (#MOB00, R & D System, Minneapolis, MD), estradiol (#DSL- 4800, Beckman Coulter, Danvers, MA), testosterone (#DSL- 4900, Beckman Coulter, Danvers, MA), insulin (#80558, Crystal Chem, Downers Grove, IL) and progesterone (#90060, Crystal Chem, Downers Grove, IL) and CRP (#1010, Alpha Diagnostics International, Antonio, TX). Samples were measured in duplicate in each assay.

Hepatic triglyceride determination—Liver triglycerides were measured using 100 mg of liver tissue and Pointe Scientific Triglyceride reagent set (#T7532-120) according to manufacturer's specifications. The sample was measured in duplicate.

RNA processing and real-time PCR—Tissue was flash frozen on dry ice then stored in -80°C until further processing. Hepatic RNA was extracted using a QIAGEN miniprep RNA kit (QIAGEN, Inc, Valencia, CA), and complementary DNA was transcribed using an iScript complementary DNA synthesis kit (Bio-Rad Laboratories, Hercules, CA).

Quantitative polymerase chain reaction was performed on a BioRad CFX96 Touch Real-Time PCR Detection System using TaqMan inventoried gene expression assays (Life Technologies, Foster City, CA). The following primer/probes were used: acylcoenzyme A amino acid N-acyltransferase 1 (#Rn01772486_m1, ACNAT1), fatty acid synthase (#Rn00569117_m1, FASN), sterol regulatory element binding transcription factor 1 (#Rn01495769_m1, SREBP1), interleukin 6 (Rn_01410330_m1, IL6), interleukin 1 β , (Rn00580432_m1, IL1 β), carnitine palmitoyltransferase 1a (#Rn00580702_m1, CPT1a) and peroxisome proliferator activated receptor alpha (Rn00566193_m1, Ppara). These were used with 10 μl reaction in the following proportions: 0.5 μl primer/probe, 5 μl master mix (#Life Technologies), 2 μl of cDNA at a concentration of 50ng/ μl and 2.5 μl of nuclease free water. Each cDNA sample was run in duplicate and statistical agreement between replicates $\pm 0.39\%$.

Paraffin embedding—Paraformaldehyde (PFA) post-fixed blocks of liver were subjected to standard paraffin-embedding and then sectioned at 5 μm on to glass slides and processed for standard hematoxylin and eosin stain.

Tissue lipid staining—For lipid staining, 10 μm PFA post-fixed liver sections were rinsed with 10% NBF, stained with Oil Red O for 10 min, and then subjected to standard hematoxylin staining and mounted on glass slides and coverslipped with an aqueous mounting media.

Statistical analyses—All statistical analyses were performed using GraphPad Prism version 6.07 (GraphPad Software, San Diego, California) USA. Differences between 2 surgeries were assessed by using paired Student's *t* test, unpaired Student's *t* test and two-tailed distribution. To observe time-wise differences, two-way repeated measures ANOVA (variables: surgical group and time) with Bonferroni *post hoc* test was used. All results are given as means $\pm$ SEM. Results were considered statistically significant when $p < 0.05$.

RESULTS

Changes in food intake and body mass

Female animals rats were ovariectomized (OVX) at 0 w and increased their body weight eating standard chow during the first 3 weeks (3 w) following OVX ($P < 0.001$) and then again when placed on a palatable high-fat diet for 3 weeks (6 w) ($P < 0.001$) (**Figure 1A**). Body fat substantially increased during the 3 weeks following OVX (3 w) ($P < 0.001$) and then also during the 3 weeks on HFD (6 w) ($P < 0.001$) (**Figure 1B**). Lean body mass significantly increased during the first 3 weeks following OVX (3 w) ($P < 0.001$) and also

during the 3 weeks of HFD induction (6 w) ($P<0.001$) (**Figure 1C**). At the beginning of the 7th week, all animals received either Sham or VSG surgery and were therein called OVX-Obese and OVX-VSG respectively. All the animals initially lost weight within the first 7 d after either procedure (**Figure 1D**). OVX-VSG animals lost significantly more weight than the OVX-Obese group during days 14 through 35 (d14-35: $P<0.001$) (**Figure 1D**). The average daily food intake was significantly reduced in the OVX-VSG group post-surgery starting at day 8 ($P<0.05$) but it approached that of the OVX-Obese group by day 35 (**Figure 1E**). Overall cumulative food intake was significantly reduced by OVX-VSG animals in comparison to OVX-Obese females starting at d 3 ($P<0.05$) (**Figure 1F**). Five weeks after surgery (i.e. at 11 w total), the OVX-VSG group showed significantly reduced body weight ($P<0.001$) (**Figure 1A**), body fat ($P<0.001$) (**Figure 1B**) but no changes in lean body mass composition as compared to before VSG surgery (**Figure 1C**).

Changes in lipid and glucose metabolism

On post-operative day 29 (POD29) animals were bled from their tail vein 1 h after lights on, they were fasted for 24 h and then bled again. In comparison to OVX-Obese animals, OVX-VSG rats showed significantly reduced triglyceride levels before the 24 h fast ($P<0.01$) (**TABLE 1**) but no significant differences in *ad libitum* plasma cholesterol or in triglyceride levels after fasting (**TABLE 1**).

On POD37, both groups of animals were fasted for 6 h and then tested for blood glucose levels. No significant difference between the groups was noted (**Table 1**). However, an intraperitoneal glucose tolerance test performed on the 6 h fasted rats showed that the OVX-Obese animals were significantly glucose intolerant in comparison to the OVX-VSG rats ($P<0.05$, T=15, 30, 45, 60) (**Figure 2A**). The reduced glucose tolerance was not due to decreased blood insulin levels (**Figure 2B**). Insulin levels did increase in both groups as a result of time ($P<0.05$) (**Figure 2B**). On POD42, animals were again fasted for 6 h prior to an insulin tolerance test (ITT). No differences in blood glucose were measured during the ITT among the two groups indicative of no insulin resistance (**Figure 2C**).

Changes in hepatic mitochondria

During POD49-52, three animals were euthanized daily in a counterbalanced fashion in order to allow for the isolation and assay of intact liver mitochondria approximately 6 h into the light cycle. Whole fresh liver was excised for examination of liver mitochondrial content and mitochondrial function. Using citrate synthase activity as the marker for mitochondria [30], the overall amount of mitochondria was found to be similar in the livers of OVX-Obese and OVX-VSG animals (**Figure 3A**). Using intact mitochondria and an NADH-generating substrate (glutamate plus malate) we found no significant differences between the OVX-Obese and OVX-VSG groups in the rate (State 3) and efficiency (RCR) of ATP production by liver mitochondria, or in the rate of proton leak across the inner membrane (State 4) (**Figure 3B**). Using fatty acid as a substrate (palmitoyl carnitine plus malate) (**Figure 3C**), there were likewise no significant differences in the rate of ATP synthesis (State 3), efficiency (RCR) or proton leak (State 4). However, the rate of uncoupled O₂ consumption using fatty acid was significantly lower in mitochondria isolated from the livers of OVX-VSG rats ($P<0.05$) (**Figure 3C**) indicating a decline in fatty acid oxidation capacity.

We prepared open, washed mitochondrial membranes (MM) from intact mitochondria for further assays of electron transfer. The rate of electron transfer from succinate to O₂ was significantly increased in the livers of OVX-VSG animals ($P<0.001$) (**Figure 4A**), while electron transfer from NADH to O₂ was not (**Figure 4B**). Assays of individual electron transfer complexes showed that this difference was not due a decline in the activity of complex I (**Figure 4C**), rather it was apparently caused by a significant increase in the activity of complex IV ($P<0.001$) (**Figure 4D**).

Changes in tissue histology, markers of liver damage, hormone levels and gene expression

Liver histology stained by H&E showed no visual differences between groups. (**Figure 5A**). Oil Red O staining showed significant reduction in lipid droplets in the OVX-VSG females in comparison to OVX-Obese (**Figure 5B**). When hepatic lipids were quantified using a colorimetric assay, the OVX-VSG group showed a significant reduction in liver triglycerides ($P<0.01$) (**Figure 5C**). Adipose tissue histology by H&E showed visible reduction in adipocyte size in OVX-VSG rats (**Figure 6**) that we did not quantify. This is in line with reduced overall fat mass composition by NMR (**Figure 1B**). Reduced levels of leptin in circulation ($P<0.05$) are associated with the overall decrease in fat mass in the OVX-VSG group (**Table 1**).

ALT and AST are circulating transaminases released by necrotic hepatocytes. Circulating ALT levels were reduced in OVX-VSG rats in comparison to OVX-Obese ($P<0.05$) (**Table 1**). There was also a trend towards reduced AST levels in the OVXVSG group ($P=0.07$) (**Table 1**).

OVX-VSG females showed a trend towards less plasma estradiol than OVX-Obese animals (**Table 1**) but there was no difference in plasma testosterone or progesterone (**Table 1**). We found no difference between circulating levels of C-reactive protein between the OVX-Obese and OVX-VSG groups (**Table 1**)

Analyses of liver gene expression by RT-PCR showed no changes in the expression of several liver genes involved in lipid metabolism and mitochondrial biogenesis, including fatty acid synthase (FASN), a critical enzyme in fatty acid synthesis, sterol regulatory element-binding protein-1 (SREBP1), a transcription factor involved in fatty acid biosynthesis, carnitine palmitoyl transferase (CPT1 α), a rate limiting enzyme of mitochondrial fatty acid β -oxidation and PGC1 α , a regulator of mitochondrial biogenesis (**Figure 5D**). Likewise, we observed no differences in either interleukin 6 (IL6) or interleukin β (IL β) mRNA expression in the liver suggesting no difference in inflammation among the two groups. We did observe a significant up-regulation of ACNAT1 expression ($P<0.05$) (**Figure 5D**), an enzyme involved in taurine conjugation of 16C fatty acids from peroxisomes and overall signaling by bile acids.

DISCUSSION

Weight changes associated with menopause and surgical menopause

Menopause results in a constellation of physiologic changes that have been well documented in human and animal models. Here we use bilateral oophorectomy (OVX) with no replacement of hormones to recapitulate the various sequelae of menopause in a rodent model. In human menopause, females gain anywhere from 2.5 kg at the low end to greater than 4.5 kg of body weight in the three years following the onset of menopause symptoms [31]. Rats maintained on chow diet after OVX surgery increase in body weight substantially in the 3 weeks following surgically-induced [32]. This study shows that the body mass gain over the intervening 3 weeks following OVX is mostly lean mass and only secondarily fat mass. Subsequent to HFD induction following week 3, the increase in body weight is primarily due to increased expansion of adipose mass. Weight changes associated with VSG during surgical menopause. Little work has been reported about the effects of bariatric surgery in women of menopausal age. One study compared laproscopic banding with RYGB in women who were 55-65 y, and found no difference in efficacy to lose weight [19]. VSG was not reported in this cohort of women. In fact, this study is the first to examine fat mass and lean mass in surgical menopausal females having received VSG. We find that VSG is sufficient to reduce body weight in OVX female rats by approximately 11%. This is similar to the effect of VSG in freely cycling female rats [26] but less than the 15.5% reduction body weight observed in male rats following VSG over a similar time frame. [28, 33]. The adiposity lost by OVX-VSG rats brought them back to the mass of adiposity achieved directly prior to placement on HFD, but VSG did not impact the adiposity that was gained during the 3 weeks of standard chow following surgical ovariectomy. The reduced body weight after VSG correlates with reduced food intake. The animals did not overeat to compensate for the body weight lost at any time. EchoMRI analysis shows that the body weight loss is fat mass and lean mass is completely preserved.

Effect of VSG on glucose metabolism

VSG surgery in surgical menopause (OVX) rats was not associated with decreased blood glucose levels after fasting in comparison to OVX-Obese, in contrast to reductions in fasting blood glucose after VSG in freely cycling females [26] and in males after VSG [28]. This may be due to differences in the degree of fasting in each of the studies. VSG surgery in the OVX rats did lead to improved glucose tolerance, as would be expected with their reduced bodyweight; this is of similar magnitude to freely cycling females that we previously reported [26]. Consistent with the finding of no change in fasting blood glucose levels, OVX-VSG animals showed no differences in fasting insulin levels as compared to obese rats without VSG. This agrees with other models using females overlaid with VSG [26, 34]. OVX-VSG rats also exhibited no difference in glucose-elicited insulin levels (T= 15 min, post dextrose injection), consistent with a reproductively-intact rat undergoing VSG [34]. Overt insulin resistance in rat models is not often observed after a short time on a saturated fat diet such as in this study. Therefore it is not surprising that there is no effect on insulin sensitivity was observed in OVX-VSG in comparison to OVX-Obese. Taken together, we find that VSG surgery improves glucose tolerance in menopausal (OVX) rats, while baseline

blood glucose levels, the glucose-elicited insulin response and whole body insulin sensitivity were not changed.

VSG and hormone levels

In humans, reproductive hormones are thought to be affected by surgical weight loss. In males having received RYGB, estradiol levels decrease significantly, while total testosterone, free testosterone and sex hormone binding globulin (SHBG) increase substantially with no changes in FSH and LH. [35]. We would anticipate similar profiles in reproductive hormones in women having undergone menopause and also having received a weight loss surgery like VSG. Surprisingly, changes in hormone levels have not been documented following surgical weight loss in women who are ovulatory with normal menses or in post-menopausal females. In our surgical menopause rat model, females have low levels of estradiol due to OVX. Following VSG surgery there is a trend towards even lower estradiol levels but no changes in testosterone or progesterone. We hypothesize that VSG leads to a further decline in estradiol due to lower production of the hormone by adipose since adipose mass decreases following VSG. Because we see no increase or reduction in lean body mass in VSG, it is not surprising that we do not see a change in testosterone levels.

VSG and hepatic steatosis

In previous studies in reproductively-intact females, we reported increased hepatic steatosis in female rats who had received VSG and then subsequently became pregnant [26]. If this phenomenon were to be recapitulated in the human population it is of grave long-term concern to women; to date this has not been studied in humans however. In this study, we wanted to determine in a menopause model whether VSG would lead to increased hepatic steatosis, as in premenopausal female rats [26] or whether VSG would lead to improvements in steatosis, as reported for male rats [6]. We found that VSG strongly lowered hepatic steatosis in surgical menopause (OVX) rats. This indicates that a decline in estradiol does not preclude an improvement in hepatic steatosis and suggests that the increased steatosis as seen in pregnant, freely-cycling female rats [26]) is possibly dependent on or influenced by high estradiol levels.

VSG and liver mitochondrial function

Since mitochondria are key to metabolism, an overview of metabolic changes associated with VSG should include an examination of mitochondrial function. For example, some studies have linked hepatic steatosis with mitochondrial dysfunction [36, 37]. Since we found that VSG relieves hepatic steatosis, might the amount of liver mitochondria decline and/or might they show a decreased capacity for fatty acid oxidation? Moreover, VSG and other bariatric surgeries alter hormonal status, which often leads to changes in mitochondrial function. Data on mitochondrial function in rat models of bariatric surgery is sparse and the effect of VSG, in particular, has not been examined. In fact, this study, to our knowledge, is the first to examine mitochondrial function after bariatric surgery of any type in *female* rats.

VSG does not alter the amount of mitochondria in liver. Citrate synthase activity, which has been demonstrated to be a reliable marker of tissue mitochondrial content [30], is the same

in OVX-Obese and OVX-VSG rats. Using substrate (glutamate plus malate) that drives electron transfer from NADH to O₂, and using the fatty acid substrate palmitoyl CoA, we found no significant differences between the OVX-Obese and OVXVSG groups in the rate (State 3) and efficiency (RCR) of ATP production by liver mitochondria, or in the rate of proton leak across the membrane (State 4). We did observe a small decline in the maximum capacity to oxidize fatty acids following VSG surgery, perhaps in response to the significant loss in fat mass in the OVX-VSG group. We conclude that the hormonal and metabolic changes associated with VSG surgery do not affect the ability of liver mitochondria to produce ATP.

While studies of mitochondria isolated from male rats after bariatric surgery have not reported on the capacity of oxidative phosphorylation to synthesize ATP, they have reported changes in the content of complex I or complex IV of mitochondria. Using blue-native gels, Guijarro *et al* [38] reported a ~40% decline in the content of complex I in skeletal muscle mitochondria following RYGB, while Verbeek *et al* [39] reported a doubling the content of complex I in liver mitochondria after RYGB. Our measurements of the rotenone-sensitive NADH→quinone activity of complex I show that its content remains unaltered in liver mitochondria after VSG surgery in OVX female rats. However, we do find that VSG is accompanied by a 20% increase in the activity of complex IV, likely due to an increase in the amount of complex IV in the membrane. An increase in complex IV content has also been observed via immunoblots in male rats following bariatric surgery. Peng and Murr [40] report a 60% increase in liver complex IV following RYGB while Pezeshki *et al* [41] report a similar increase in skeletal muscle following ileal transposition.

There exists evidence for less production of reactive oxygen species (ROS) in liver mitochondria following bariatric surgery in male rats [39, 40]. We measured the percent of electrons that “leak” to O₂ to produce superoxide during electron transfer from succinate to O₂ in washed mitochondrial membranes prepared from OVX-Obese and OVX-VSG female rats. In fact, superoxide production was ~30% lower in the OVXVSG group, but the difference (data not shown) fell short of significance. Nonetheless, we note that the increase in complex IV activity in OVX-VSG female rats is likely to lower the production of mitochondrial ROS. Mitochondrial ROS production takes place during electron transfer as one-electron intermediates generated by complexes I and III occasionally reduce O₂ to superoxide [42]. Superoxide then goes on to form hydrogen peroxide and the potent hydroxyl radical, which causes protein carbonylation and lipid peroxidation. Increased complex IV activity, in the absence of increased complex I activity (the situation in our OVX-VSG animals), will leave the electron transfer chain of mitochondria more oxidized. This, in turn, decreases the lifetime of the O₂-reducing intermediates in complexes I and III and lowers the production of superoxide [43].

Conclusion

VSG is increasingly being sought after in women of all ages. At present about 40% of the total number of patients seeking bariatric surgery are women of menopausal age. Our present data highlight that VSG does work successfully in a rodent model of menopause and begins to fill in the lack of knowledge about bariatric surgery with menopause. We find that

VSG results in physiologic improvements in body weight, glucose tolerance and hepatic lipids. Liver mitochondrial function is maintained and an increase in complex IV may decrease the production of mitochondrial ROS. Further we provide evidence that the hepatic lipid steatosis observed in post-partum female rats does not occur in ovariectomized female rats; in fact hepatic lipids are improved. Future work is necessary to determine if these results are recapitulated in humans. Specific work is also required to understand liver function and the resolution of liver disease after VSG, in cohorts separated by age and reproductive status.

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ABBREVIATIONS

ACNAT1	acyl-coenzyme A amino acid N-acyltransferase 1
ALT	alanine aminotransferase
AST	aspartate aminotransferase
CPT1a	carnitine palmitoyltransferase 1a
DNA	deoxyribonucleic acid
h	hours
HFD	high fat diet
IL1β	interleukin 1 β
IL6	interleukin 6
MetS	metabolic syndrome
min	minutes
NAFLD	non-alcoholic fatty liver disease
OVX	ovariectomy
PFA	paraformaldehyde
PPAR1	peroxisome proliferator activated receptor alpha
RCR	respiratory control ratio
RNA	ribonucleic acid
RYGB	roux en y gastric bypass

SEM	standard error of the mean
T2DM	type 2 diabetes mellitus
VSG	vertical sleeve gastrectomy
y	years

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Figure 1.

Body weight, body composition and food intake following VSG. A. Body weight in female rats at the start of the study (0 w), three weeks after ovariectomy (3w), following 3 weeks HFD induction (6 w) and then 5 weeks after VSG or sham surgery (11 w). B. Fat mass in female rats at the start of the study (0 w), after ovariectomy (3w), following HFD induction (6 w) and then following VSG or sham surgery (11 w). C. Lean mass in female rats at the start of the study (0 w), after ovariectomy (3w), following HFD induction (6 w) and then following VSG or sham surgery (11 w). D. Body weight curve after VSG or sham surgery. E. Average daily food intake in kCal during the first 5 weeks after VSG or sham surgery F. Cumulative food intake in kCal during the first 5 weeks after sham or VSG surgery (N=12,9)

*P < 0.05, **P < 0.01, ***P < 0.001. Data are presented as mean $\pm$ SEM.

Figure 2. Glucose and insulin sensitivity parameters following VSG. A. Intraperitoneal glucose tolerance test over 2 h period. (N=11,9) B. Circulating insulin levels after 6 h fast (baseline) and 15 min after dextrose gavage (N=8,8) . C. Insulin tolerance test curves (N=8,5). *P < 0.05. Data are presented as mean ±SEM.

Figure 3.
A. Citrate synthase activity per mg total liver protein as a marker for mitochondrial content
B. Respiration of 20 mM glutamate plus 5 mM malate and C. 40 μM palmitoyl carnitine plus 5 mM malate, in intact liver mitochondria as described in Methods. *P < 0.05. (N=3,4)
Data are presented as mean± SEM.

Figure 4.

Assays with washed, open mitochondrial membranes as described in Methods. A. Succinate to O₂ B. NADH to O₂. C. Complex I activity D. Complex IV activity. ***P < 0.001, Student's t test. (N=8,5) Data are presented as mean ± SEM.

Figure 5.

Hepatic histology and gene expression following VSG and Sham surgeries. A. H&E staining in liver. Bar = 100μm (N=5,4). B Oil Red O staining in liver. C. Hepatic triglyceride content measured by colorimetric assay (N=12,9). D. Hepatic gene changes by real-time PCR following VSG and Sham surgeries (N=12,9). *P < 0.05, **P < 0.01. Data are presented as mean ±SEM.

Figure 6.
H&E staining in adipose tissue following VSG and Sham surgeries, Bar = 100 μ m (N=5, 4)

Table 1

Plasma analyte measurements

Analyte		OVX-Obese	OVX-VSG	P
6 h fasting glucose	(mg/dL)	124 ± 3.77	119.4 ± 3.64	0.3975
<i>ad lib</i> fed plasma triglycerides	(mg/dL)	224.31 ± 32.21	117.24 ± 18.24 **	0.0013
24 h fasting plasma triglycerides	(mg/dL)	87.973 ± 13.60	80.14 ± 9.346	0.3682
<i>ad lib</i> fed plasma cholesterol	(mg/dL)	98.15 ± 4.71	90.79 ± 3.53	0.5156
24 h fasting plasma cholesterol	(mg/dL)	93.37 ± 4.46	86.24 ± 5.41	0.5485
aspartate aminotransferase (AST)	IU/L	203 ± 27.60	114.20 ± 21.51 *	0.0293
alanine transaminase (ALT)	IU/L	42.67 ± 4.68	31.84 ± 3.49	0.0903
leptin	(pg/mL)	596.22 ± 89.96	264.09 ± 59.13 *	0.0128
estradiol	(pg/mL)	5.68 ± 0.53	4.20 ± 0.478	0.0944
free testosterone	(pg/mL)	0.10 ± 0.01	0.11 ± 0.004	0.5164
progesterone	(ng/mL)	56.89 ± 8.44	58.52 ± 17.42	0.9253
c-reactive protein (CRP)	(mg/mL)	222.30 ± 28.14	183.50 ± 15.64	0.2776

Data are presented as mean ± SEM. (N=12, 9)

*
p < 0.05

**
p < 0.01.