

The effects of voluntary exercise on oocyte quality in a diet-induced obese murine model

Anna L Boudoures, Maggie Chi, Alysha Thompson, Wendy Zhang and Kelle H Moley

Division of Basic Science Research, Department of Obstetrics and Gynecology, Washington University in St. Louis School of Medicine, 425 South Euclid Avenue, Campus Box 8064, St Louis, Missouri 63110, USA

Correspondence should be addressed to K H Moley; Email: moleyk@wustl.edu

Abstract

Obesity negatively affects many aspects of the human body including reproductive function. In females, the root of the decline in fertility is linked to problems in the oocyte. Problems seen in oocytes that positively correlate with increasing BMI include changes to the metabolism, lipid accumulation, meiosis, and metaphase II (MII) spindle structure. Studies in mice indicate that dietary interventions fail to reverse these problems. How exercise affects the oocytes has not been addressed. Therefore, we hypothesized an exercise intervention would improve oocyte quality. Here we show that in a mouse model of an exercise, intervention can improve lipid metabolism in germinal vesicle (GV) stage oocytes. Oocytes significantly increased activity and transcription of the β -oxidation enzyme hydroxyacyl-coenzyme A dehydrogenase in response to exercise training only if the mice had been fed a high-fat diet (HFD). An exercise intervention also reversed the lipid accumulation seen in GV stage oocytes of HFD females. However, delays in meiosis and disorganized MII spindles remained present. Therefore, exercise is able to improve, but not reverse, damage imparted on oocytes as a result of an HFD and obesity. By utilizing an exercise intervention on an HFD, we determined only lipid content, and lipid metabolism is changed in GV oocytes. Moving forward, interventions to improve oocyte quality may need to be more targeted to the oocyte specifically. Because of the HFD-induced deficiency in β -oxidation, dietary supplementation with substrates to improve lipid utilization may be more beneficial.

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Introduction

Approximately 25% of individuals in the western world are obese, and the worldwide prevalence of obesity is predicted to continue increasing (Kelly *et al.* 2008). Obesity predisposes individuals to many diseases including type 2 diabetes, cardiovascular disease, and stroke (Swift *et al.* 2014). In addition to impairing overall health, obesity has been linked to subfertility (Bellver *et al.* 2010). This subfertility can likely be traced to the oocyte, as studies of oocytes obtained from women undergoing IVF demonstrated that oocytes from obese women are often apoptotic and meiotically delayed (Wittemer *et al.* 2000, Metwally *et al.* 2007).

To better understand the mechanisms causing decreased oocyte quality, many labs have modeled diet-induced obesity in mice by feeding a high-fat diet (HFD). These studies have shown that HFD affects oocyte meiotic maturation, ovulation, and fertilization and leads to embryos with restricted growth and brain abnormalities (Minge *et al.* 2008, Jungheim *et al.* 2010, Luzzo *et al.* 2012). Additionally, when blastocysts or two-cell embryos fertilized in HFD mice were transferred to control recipients, the resulting fetuses still had restricted growth and brain abnormalities, confirming

that the defects arose from the oocytes and not the uterine environment (Sasson *et al.* 2014). Several papers have detailed the negative effects of HFD on oocyte quality in mice. After exposure to an HFD for only 4 weeks, oocytes of HFD mice show increased lipid accumulation, lipotoxicity, and endoplasmic reticulum stress (Wu *et al.* 2010). Additionally, compared to controls, HFD mice ovulate a significantly higher proportion of oocytes with disorganized meiosis II spindles (Luzzo *et al.* 2012). Finally, HFD oocytes have a significant increase in mitochondrial damage, including the appearance of large vacuoles and ruptured membranes, and a significant decrease in citrate production, suggesting defects in citric acid cycle metabolism (Luzzo *et al.* 2012).

Previously, our lab attempted to ameliorate HFD-induced oocyte damage by returning the mice to a control diet. Although overall physiology improved, including weight loss and return to normal glucose tolerance, the benefits were not paralleled in the oocytes (Reynolds *et al.* 2014). In humans, exercise confers many physiological benefits including decreased risk of developing cardiovascular disease, type 2 diabetes, and stroke, even in the absence of weight loss (Swift *et al.* 2014). Additionally, several rodent studies revealed

that offspring of exercised dams were significantly healthier than offspring of sedentary dams (Bradley *et al.* 2008, Wagener *et al.* 2012, Carter *et al.* 2013, Vega *et al.* 2013). Finally, HFD female rats that were allowed to exercise had significant fertility improvements despite the lack of other physiological improvements (Vega *et al.* 2013).

Because the reproductive consequences of obesity are likely rooted at the oocyte level, and exercise is beneficial to both mother and offspring, we hypothesized that even without a change in diet, the physiological benefits of exercise would be reflected in oocytes. To test this hypothesis, we allowed HFD females to voluntarily exercise for 6 weeks and compared their oocytes to those of sedentary HFD mice and sedentary mice on a control diet. We assayed oocytes for changes in lipid accumulation, mitochondrial damage, and hydroxyacyl-coenzyme A dehydrogenase (HADHA) activity (a fatty acid oxidation enzyme) and recorded the percentage of oocytes ovulated either prior to meiosis II arrest or with deformed spindles. Our data revealed that exercise was able to improve, but not entirely reverse, the negative effects of HFD on oocytes.

Materials and methods

Animals and diet

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the IACUC-accredited Animal Studies Committee of Washington University School of Medicine (study #20120051). Female C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) were fed either an HFD (Test Diet 58R3, 58% fat energy) or a standard mouse chow diet (CD) (Lab Diet 5053, 13% fat energy) *ad libitum* from 4 weeks of age.

Exercise exposure

For the first 6 weeks of the study (age 4–10 weeks), mice were housed four per cage (feeding period). Mice were then moved to standard rat cages and housed eight mice per cage either with or without *ad libitum* exposure to exercise wheels (exercise exposure period) for 6 weeks. Mice remained on their respective HFD or CD during this period.

Body composition analysis

No more than 12 h before sacrifice, eight mice from each experimental group were subjected to body composition analysis to determine absolute fat and lean tissue mass by magnetic resonance imaging (EchoMRI 3-in-1; Echo Medical Systems, Houston, TX, USA). Two or three measurements were taken per mouse to ensure instrument precision, and values for individual mice were averaged for statistical analyses.

Glucose tolerance test

Eight to nine mice per experimental group were fasted for 12 h and then administered an i.p. injection of glucose (2 mg/g body weight) with a 30×½ gauge needle. Blood glucose was monitored by readings of a small (2–3 µl) drop of tail vein blood with a one-touch Ultra Glucose Monitoring glucometer and test strips (Johnson and Johnson, New Brunswick, NJ, USA) at 15, 30, 60, 90, and 120 min after injection. Mice were then returned to their respective diets.

Serum triglyceride, glucose, and insulin levels

Eight mice in each experimental group were fasted for 4 h before blood was collected by puncturing the facial vein with a 25×5/8 gauge needle. Approximately 50 µl blood was collected in BD Microtainer Serum Separator Tubes (Franklin Lakes, NJ, USA). At the same time as facial vein bleed, fasting blood glucose was measured by readings of a small (2–3 µl) drop of tail vein blood with a one-touch Ultra Glucose Monitoring glucometer and test strips (Johnson and Johnson). After blood collection, mice were returned to their respective diets. Tubes were incubated for 30 min at room temperature (RT) and spun at 11 753 g for 10 min at RT. Serum was collected on ice and stored at –20 °C until analysis. The Infinity Triglycerides Liquid Stable Reagent Kit (Thermo Scientific, Waltham, MA, USA) was used according to the manufacturer's protocol to measure triglycerides. The Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Downers Grove, IL, USA) was used according to the manufacturer's protocol to measure serum insulin levels. Each serum sample was tested in duplicate for precision, and values were averaged prior to statistical analysis.

The homeostatic model assessment of insulin resistance (HOMA1–IR) is a validated measure of IR that is calculated using the values listed in Table 1 using the following formula:

$$\text{HOMA1-IR} = \frac{(\text{FSI} \times \text{FSG})}{22.5},$$

where fasting serum insulin (FSI, mU/l) and fasting serum glucose (FSG, mmol/l) (Matthews *et al.* 1985).

4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene 493/503 staining of germinal vesicle stage oocytes

Three mice from each experimental condition were administered an i.p. injection of 10 IU pregnant mare serum gonadotropin (PMSG) 46–48 h prior to sacrifice. Ovaries were removed and placed in M2 media + 4 mM hypoxanthine (Sigma–Aldrich). Large antral follicles were punctured with a 29×½ gauge needle, and cumulus cells were removed by multiple passes through a glass pipette. Oocytes were then fixed in 4% paraformaldehyde (Sigma–Aldrich) diluted in PBS + 1% polyvinylpyrrolidone (PVP; Sigma–Aldrich), washed twice, and transferred to a 1 µg/ml solution of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene 493/503 (BODIPY; Invitrogen) + 1% PVP for 1 h at RT in the dark. After two washes in PBS + 1% PVP, oocytes were mounted in VectaShield (Vector Labs, Burlingame, CA, USA) and visualized by using an Olympus

Table 1 Serum insulin and blood glucose levels for 12-week old mice.

	CD exercise	CD sedentary	HFD exercise	HFD sedentary
Serum insulin (ng/ml)				
Mouse 1	0.48	0.33	0.74	0.72
Mouse 2	0.46	0.42	1.06	1.73
Mouse 3	0.76		1.53	0.28
Mouse 4	0.53	0.55	1.07	0.87
Mouse 5	0.53	0.41	2.05	0.91
Mouse 6	0.50		1.05	1.52
Mouse 7	0.50	0.41	0.69	1.17
Mouse 8	0.78	0.33	0.62	1.06
Blood glucose (mmol/l)				
Mouse 1	9.55	5.83	7.22	7.10
Mouse 2	6.05	5.83	6.16	8.49
Mouse 3	5.83	5.94	5.99	8.44
Mouse 4	6.27	4.72	8.49	8.55
Mouse 5	5.38	5.72	8.88	7.83
Mouse 6	5.99	5.88	6.66	8.66
Mouse 7	6.11	4.33	9.55	7.44
Mouse 8	6.66	5.11	8.44	9.82

laser-scanning confocal microscope at 20× magnification. All images were taken as a single optical section through the plane containing the germinal vesicle (GV) at the same laser power and gain settings and adjusted by using the same gain and contrast adjustments in Adobe Photoshop CS6. For analysis, the mean gray value of pixels in an individual oocyte was measured in a minimum of 25 oocytes/group.

Transmission electron microscopy

Denuded GV stage oocytes from three mice per experimental group were collected as described for BODIPY staining and fixed in 2% paraformaldehyde+2.5% glutaraldehyde (Polysciences, Warrington, PA, USA) in 100 mM cacodylate buffer (Sigma–Aldrich), pH 7–7.2, for 1 h at RT. Oocytes were embedded in 2% agarose and washed 3×30 min in 100 mM cacodylate buffer at RT. Agarose-embedded oocytes were then fixed in 1% OsO₄ (Electron Microscopy Sciences, Hartfield, PA, USA) in 100 mM cacodylate buffer for 1 h at RT. Oocytes were washed thoroughly in distilled water, stained in 1% uranyl acetate in dH₂O for 1 h at RT, dehydrated in a series of ethanol washes, and then washed in 100% ethanol at RT. Next, oocytes were washed twice in propylene oxide (Electron Microscopy Sciences) and then infiltrated with resin by using the Eponate 12 Kit (Ted Pella, Redding, CA, USA). After infiltration, blocks were embedded, cured, and then sectioned with a Leica Ultracut UCT Ultramicrotome (Leica Microsystems, Inc., Bannockburn, IL, USA). Sections (90 nm) were stained with uranyl acetate and Reynolds' lead citrate before viewing at 2000×–15 000× magnification with a JEOL 1200 EX Transmission Electron Microscope (JEOL USA, Inc., Peabody, MA, USA) equipped with an AMT eight megapixel digital camera (Advanced Microscopy Techniques, Woburn, MA, USA). Images taken at 15 000× magnification were blinded and analyzed by three independent observers for mitochondrial shape, the number of lipid droplets in each image, and the number of rose petal mitochondria in each image.

HADHA type 2 enzymatic activity

Denuded GV stage oocytes were collected from three mice per group as described for BODIPY staining. Individual oocytes were frozen on glass slides in isopentane equilibrated with liquid nitrogen and freeze-dried overnight under vacuum at –35 °C. In total, 15 individual oocytes were extracted under nanoliter volume and used for analysis of levels of HADHA activity by enzyme-linked cycling assays as previously described (Chi *et al.* 2002).

Quantitative real-time PCR

Three GV oocytes per mouse were collected from six mice per experimental group as described for BODIPY staining. Oocytes were denuded and washed in 50 mg/ml nuclease-free BSA (Invitrogen) on ice. Oocytes were then transferred to the single cell lysis solution with single cell DNase I solution (both parts of the Single Cell-To-Ct Kit (Ambion, Austin, TX, USA)). All steps of RNA isolation and RT followed the manufacturer's protocol. Real-time PCRs were carried out on a 7500 fast real-time PCR system. Expression of carnitine palmitoyltransferase 2 (*Cpt2*), *Hadh*, aconitase 2 (*Aco2*), isocitrate dehydrogenase 3b (*Idh3b*), and *Actb* were measured by using the inventoried TaqMan Gene Expression Assays (*Cpt2*: Mm00487205_m1; *Hadh*: Mm00492535_m1; and *Actb*: Mm00607939_s1; Life Technologies). Expression of genes for each diet was normalized to *Actb* based on its utilization in prior publications as a reference gene in oocytes, and its expression remains stable throughout meiosis (Bachvarova *et al.* 1985).

Spindle and chromosome imaging of metaphase II stage oocytes

Four to five mice from each of the four experimental conditions were i.p. injected with 10 IU of PMSG (Sigma–Aldrich) and then with 5 IU of human chorionic gonadotropin (hCG; Sigma–Aldrich) 48 h after PMSG. Mice were sacrificed by cervical dislocation 12–14 h after hCG administration, and the cumulus–oocyte complexes (COCs) were collected from the ampullae. Cumulus cells were removed with a brief wash in 1 mg/ml hyaluronidase in M2 media (Sigma–Aldrich). Oocytes were then fixed for 30 min in 4% paraformaldehyde in PBS+1% BSA (Sigma–Aldrich). Oocytes were transferred to a permeabilization solution (0.5% Triton X-100 in PBS) for 20 min and then blocked with PBS+0.1% Tween-20+0.01% Triton X-100+1% BSA for 1 h. Oocytes were stained with FITC-conjugated anti-tubulin antibody (1:500; Sigma–Aldrich) overnight at 4 °C, washed three times, and then co-stained with TOPRO-3 iodide (1:500; Invitrogen) for 10 min. Oocytes were washed twice in PBS+0.1% Tween-20+0.01% Triton X-100, mounted in VectaShield, and visualized by using an Olympus laser-scanning confocal microscope at 20× magnification. Images were blinded and analyzed by three independent observers for meiotic stage (metaphase II (MII) vs prophase or anaphase), spindle shape (normal, barrel shaped vs deformed), and apoptosis.

Results

Exercise training improves some aspects of whole-body physiology

Previously, our lab and others have shown that mice maintained on a HFD became obese and had damaged oocytes. To determine whether exposure to voluntary exercise could reverse any of the defects, we exposed mice to either HFD or control CD for 12 weeks beginning at 4 weeks of age. During the last 6 weeks, half of each diet cohort had access to an exercise wheel 24 h a day, based on the experimental design of Schulkey *et al.* (exercise exposure period). Mice were filmed for a 24-h period to confirm frequent use of wheels (data not shown). We found that HFD exposure caused significant weight gain that was not reversible by exercise ($n=32/\text{group}$, Fig. 1A). However, compared to their sedentary counterparts of the same age, the cohort of HFD exercising mice had significantly less fat mass and unchanged lean mass ($n=8/\text{group}$, Fig. 1B and C). This significant change in fat mass was likely not reflected in total body mass because of the differences in sample size. HFD mice had significantly higher IR as measured

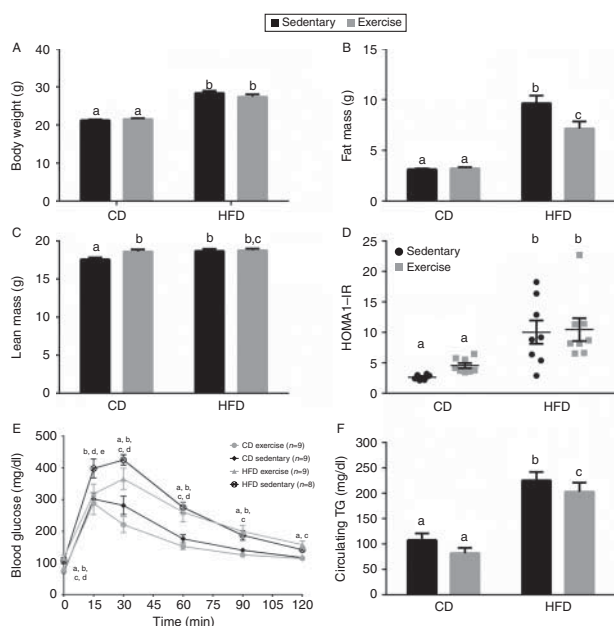


Figure 1 Body weights and body composition and metabolic parameters of mice. (A) Average body weights, (B) fat mass, and (C) lean mass as measured by magnetic resonance imaging. (D) homeostatic model assessment of insulin resistance (HOMA1-IR) calculated as described in 'Materials and methods' section. (E) Glucose tolerance test, (F) fasting serum triglycerides. (A, B, C, D, E and F) Different letters designate statistical significance between groups whereas same letter indicates no significance between groups, $P < 0.05$. (E) Letters above each time point represent statistical significance between groups at that time as follows: a, CD exercise vs HFD exercise; b, CD exercise vs HFD sedentary; c, CD sedentary vs HFD exercise; d, CD sedentary vs HFD sedentary; and e, HFD exercise vs HFD sedentary, $P < 0.05$. All data was analyzed by two-way ANOVA with Tukey-Kramer correction for multiple comparisons. CD, chow diet; HFD, high-fat diet.

by the HOMA-IR, which was not reversed by exercise (Fig. 1D). Additionally, the range of insulin sensitivity was much greater in HFD mice than CD mice. Allowing mice to exercise while maintaining a HFD was able to cause a trend toward a restoration of glucose tolerance, especially at earlier time points (see 15-min time point in Fig. 1E). Exercise was able to significantly decrease triglyceride levels in HFD females, but triglyceride levels were still higher than in CD mice; exercise did not significantly change triglyceride levels in CD mice (Fig. 1F). Therefore, our results confirm previously published findings that exercise alone cannot improve body weight, but some physiological parameters are improved by exercise (Kelly *et al.* 2011, Carter *et al.* 2013, Vega *et al.* 2013, Stanford *et al.* 2014).

Changes in oocyte lipid accumulation

Human obesity causes an increased accumulation of triglycerides in the follicular fluid surrounding the oocyte in a maturing ovarian follicle, and mouse oocytes exposed to this lipid-rich environment also contain excess lipids (Jungheim *et al.* 2011, Yang *et al.* 2012, Dunning *et al.* 2014a,b, Valckx *et al.* 2014a,b). Thus, given that exercising HFD mice had lower serum triglycerides than their sedentary peers, we hypothesized that these mice would also have decreased lipid accumulation in their maturing oocytes. To test this, oocytes were collected from superovulated females at the GV stage, stained with the fluorescent neutral lipid dye BODIPY 493/503, and imaged by confocal microscopy (Fig. 2A). Measurements of the mean gray value of the fluorescence of individual oocytes revealed that, as expected, oocytes from sedentary HFD females contained more lipid than oocytes from CD females. However, oocytes from exercised HFD females contained the same amount of lipid as those from sedentary CD females. Surprisingly, oocytes from exercised CD mice contained the most lipid of any of the groups (Fig. 2A and B).

We also examined lipid accumulation by counting lipid droplets in transmission electron microscopy (TEM) images of GV oocytes. Consistent with the BODIPY results, oocytes from sedentary HFD females contained more lipid droplets than those from exercised HFD mice or either of the CD groups (Fig. 2C and D). From these data, we conclude that exercise is able to reverse the lipid accumulation phenotype induced by exposure to HFD.

Oocyte metabolism is affected by changes in diet and exercise

Oocytes rely heavily on carbohydrates supplied by cumulus cells to generate ATP and fuel maturation and fertilization (Rieger & Loskutoff 1994). In addition,

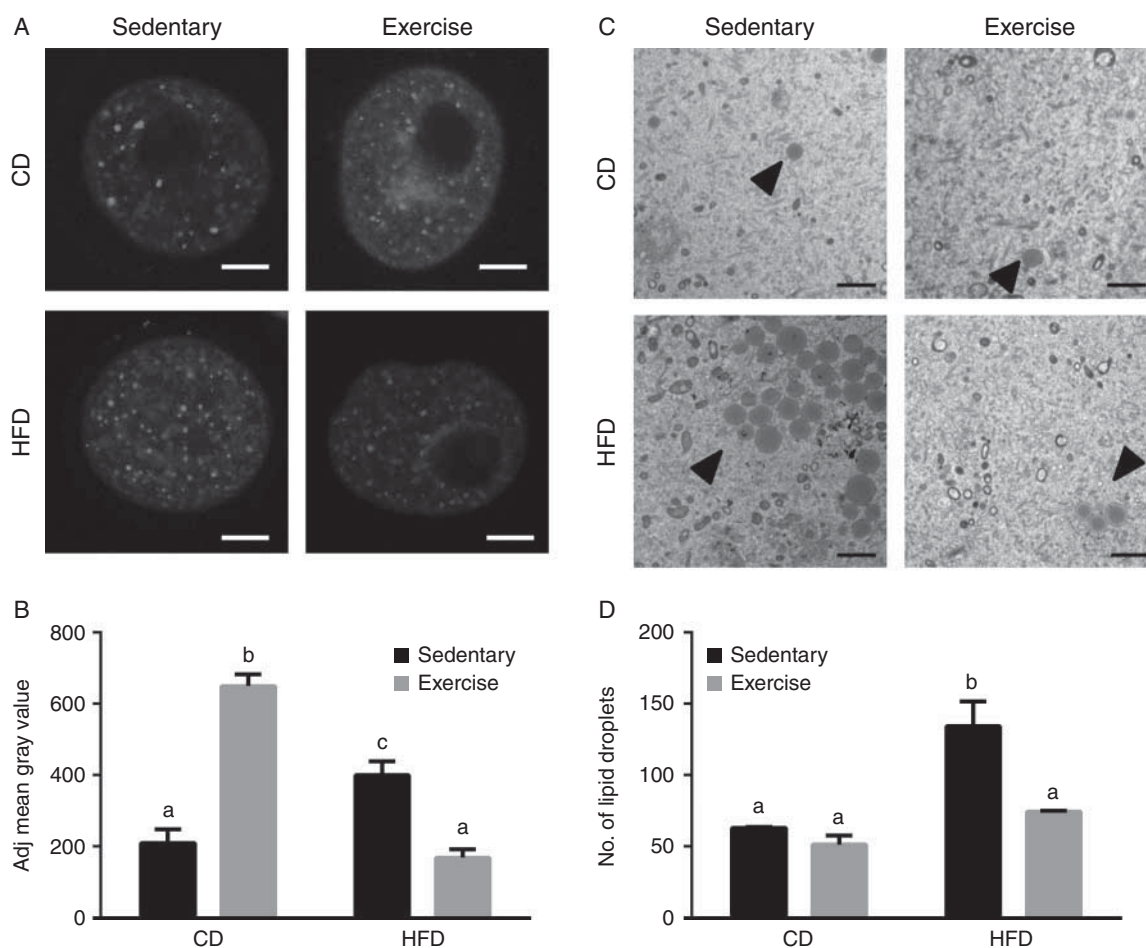


Figure 2 Lipid droplet accumulation in germinal vesicle stage oocytes in response to diet and exercise training. (A) Representative images of oocytes from BODIPY 493/503 staining. All images were taken at the same keeping confocal settings identical, scale bars: 25 μm . (B) Mean relative gray value of fluorescence of individual oocyte area measured using ImageJ (Rasabanad, WS). (C) Representative images of sections of oocytes taken by transmission electron microscopy at 2000 \times magnification, scale bars: 2 μm . Black arrowheads indicate lipid droplets. (D) Total number of lipid droplets counted in images from each group. Different letters designate statistical significance and same letter indicates no significance between groups, $P < 0.05$; two-way ANOVA with Tukey–Kramer correction for multiple comparisons.

maturing oocytes metabolize lipids by the fatty acid oxidation pathway within the mitochondria (Dunning *et al.* 2010, 2014b). Thus, the significant increase of lipids in the GV oocytes of sedentary HFD females led us to ask whether these oocytes have changes in their mitochondrial metabolism. First, the significant changes in lipid accumulation after exercise exposure led us to hypothesize that there may be changes to fatty acid metabolism. To test this, we used enzymatic cycling to measure activity of HADHA in individual GV stage oocytes. HADHA is a key enzymatic subunit of the mitochondrial trifunctional protein, which catalyzes three steps of fatty acid β -oxidation (Houten & Wanders 2010). We found that oocytes from both cohorts of HFD females had significantly lower HADHA activity than oocytes from both cohorts of CD females. Although exercise was not able to increase the level of HADHA activity in HFD oocytes to the levels observed in CD

oocytes, oocytes from exercised HFD and CD mice had significantly higher HADHA activity than those of their sedentary counterparts (Fig. 3A).

To determine if this activity change was reflected in ATP production, we next measured ATP content of individual GV oocytes using enzymatic cycling assays. However, we observed significantly lower ATP only in HFD exercising mice (Fig. 3B). Lower ATP levels contradict the positive effects of exercise on HADHA activity. Fatty acid oxidation produces 14 molecules of ATP for every two carbons in a carbon chain (e.g. 104 molecules of ATP for a single molecule of palmitate). Given the efficiency of this reaction, increased HADHA activity would be expected to increase ATP (Garrett & Grisham 2013). To decide if the contradiction of HADHA activity and ATP production could be explained by changes to other metabolic pathways, we measured citrate levels in oocytes (Fig. 3C). Citrate

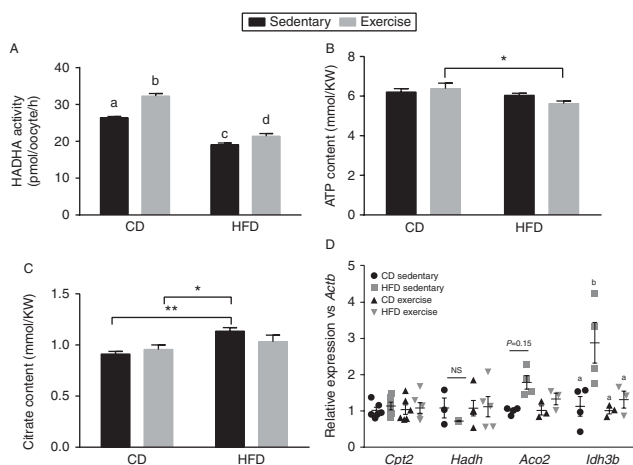


Figure 3 Metabolic enzyme activity, metabolite levels, and transcript levels are altered by diet and exercise. (A) HADHA enzymatic activity was measured in individual GV stage oocytes ($n=15$ oocytes/group). Two-way ANOVA with multiple comparisons, different letters represent statistical significance between groups, $P<0.001$. (B) ATP and (C) citrate content of GV stage oocytes as measured by enzymatic cycling assay ($n=45$ oocytes/group). Two-way ANOVA with multiple comparisons; $*P<0.05$ and $**P<0.01$. (D) Relative abundance of *Cpt2*, *Hadh*, *Idh3b*, and *Aco2* transcripts; three oocytes per mouse were collected from six individual mice per experimental condition, and relative abundance of transcripts was calculated using the $\Delta\Delta C_T$ method, with *Actb* as the reference gene. Two-way ANOVA with multiple comparisons; for *Idh3b*: a, no change between groups and b, $P<0.001$ for *Hadh* sedentary vs exercise. For *Aco2*, the difference was not statistically significant but is trending toward significance ($P=0.015$).

was significantly higher only in oocytes from HFD sedentary females. These results are both surprising and conflicting to both the activity of HADHA and ATP levels of oocytes.

Although the data are conflicting, these data agree with transcript abundance of TCA cycle enzymes and HADHA; HFD sedentary mice have significantly higher levels *Idh3b*, and levels of *Aco2* are increased, but the results did not reach statistical significance ($P=0.15$), two enzymes involved in the TCA cycle (Fig. 3D). This is likely due to increased citrate abundance. Similarly, the transcript abundance of fatty acid oxidation enzyme HADHA is significantly decreased only in HFD sedentary oocytes. This is reflected in the significant decrease in HADHA activity and lipid droplet accumulation also observed in HFD sedentary oocytes (Figs 2 and 3A respectively).

Mitochondrial ultrastructure is changed by exercise exposure

Because metabolite and qPCR data indicated changes to mitochondrial pathways, but could not fully explain the changes to lipid accumulation observed in oocytes from HFD sedentary and exercise mice, we wondered whether mitochondrial damage previously reported

persisted or was changed by exercise (Wu *et al.* 2010, Luzzo *et al.* 2012). To address this question, we used TEM to examine mitochondrial ultrastructure in GV stage oocytes. Whereas oocyte mitochondria are normally round, the appearance of elliptical and dumbbell-shaped mitochondria suggests the occurrence of fission and fusion (Friedman & Nunnari 2014). We found that oocytes from sedentary HFD females had a higher proportion of elliptical and dumbbell-shaped mitochondria than did oocytes from exercising HFD mice or CD mice (Fig. 4A and B).

Another morphological measure of mitochondrial health is the appearance of so-called 'rose petal' mitochondria (see examples in Fig. 4C). These occur when there are deficiencies in oligomerization of the F_1F_0 -ATP synthase in the mitochondrial inner membrane (Arselin *et al.* 2003). To assess presence of rose petal mitochondria in the four cohorts of mice, we collected GV oocytes from three mice from each group and subjected them to TEM. Images were coded and scored by three independent blinded observers. We found that oocytes from sedentary HFD mice had significantly more rose petal mitochondria than did oocytes from CD mice. However, oocytes from exercised HFD mice had similar numbers of rose petal mitochondria as CD mice (Fig. 4D). These results suggest that oocyte mitochondrial damage that occurs as a result of HFD exposure can be compensated for by exercise.

MII spindle structure is unchanged by exercise

Exposure to a HFD causes multiple abnormalities in oocytes including disorganized spindle structure in the MII oocyte, aberrant segregation of chromosomes, and delayed meiotic maturation (Luzzo *et al.* 2012). Lipid accumulation was also linked to delayed oocyte maturation and increased apoptosis at the MII stage (Wu *et al.* 2010). Consistent with these reports, we observed a greater proportion of delayed oocytes in HFD mice than in CD mice (CD sedentary=15%; CD exercise=7% vs HFD sedentary=38%; and HFD exercise=32%; Fig. 5). However, the proportion of normal, meiotically mature oocytes from exercised HFD mice did not differ greatly from those of sedentary HFD mice (HFD exercise=40% and HFD sedentary=32%; Fig. 5).

Discussion

Here, we report that exercise brought about moderate change to female physiology and a significant decrease in lipid accumulation in the oocytes of HFD females. This may be due, in part, to increased activity of the fatty acid oxidation enzyme HADHA. However, these positive changes did not negate the effects of HFD on spindle morphology and meiotic maturation at ovulation.

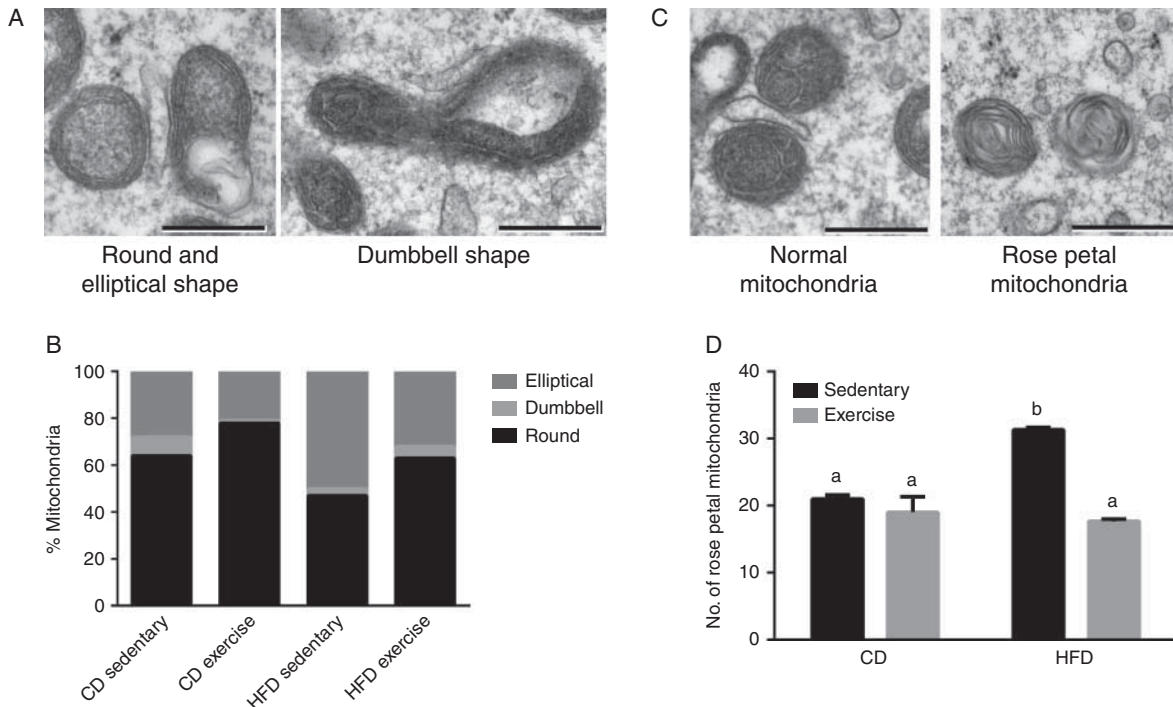


Figure 4 Transmission electron microscopy of GV stage oocytes. (A) Representative image of round (normal), elliptical, and dumbbell-shaped oocyte mitochondria. (B) Percentages of shape of mitochondria from each cohort of mice ($n=7-9$ oocytes/cohort). (C) Representative image of normal oocyte mitochondria and rose petal mitochondria. (D) Comparison of the number of rose petal mitochondria counted in oocytes from each cohort ($n=7-9$ oocytes/cohort), two-way ANOVA with multiple comparisons, different letters designate statistical significance and same letter indicates no significance between groups, $P<0.05$. All images are at $15\ 000\times$ magnification, scale bars: 500 nm.

We found that, in female mice, voluntary exercise resulted in moderate physiological benefits and no significant total body weight loss. This is not surprising for three reasons. First, in a study of mice that were forced to exercise, female mice had a much smaller decrease in total body mass and fat mass than their male counterparts (Kelly *et al.* 2010). Secondly, the mice in our study voluntarily exercised, likely at lower levels than those in the forced exercise study. Although we did not control for the exact amounts of exercise each female received, our experimental design was based off of a study that showed moderate voluntary exercise in HFD females which was beneficial in the reversal of congenital heart disease in their offspring (Schulkey *et al.* 2015). Finally, a weak relationship between exercise and weight loss has also been reported in humans (Swift *et al.* 2014).

Although body weight was largely unchanged by exposure to exercise, the lipid content of oocytes from HFD females was decreased, as shown by both our BODIPY and EM data. Although we saw more lipid in the oocytes of exercising CD mice than in their sedentary counterparts, our contrasting EM data suggest that this was due to experimental error. Oocytes were used from three mice and pooled prior to staining, thus contribution from any one mouse is not possible to determine.

Our data are consistent with previous findings that lipid accumulates in oocytes in the presence of increased substrate availability both *in vivo* (Wu *et al.* 2010, Luzzo *et al.* 2012) and *in vitro* (Jungheim *et al.* 2011), but the mechanism of lipid uptake by oocytes is still under investigation.

The exercise-induced reductions in lipid content of oocytes we observed in HFD mice might, in part, be explained by the significant decrease in circulating serum triglycerides in the mice. Previous research in both mice and humans has shown that serum levels of triglycerides correlate with triglyceride levels in both the ovarian follicle and the oocyte (Wu *et al.* 2012, Yang *et al.* 2012).

The accumulation of lipids within oocytes of HFD females and the reductions we recorded after exercise exposure may also be partially explained by changes to expression of TCA cycle enzymes and fatty acid β -oxidation. We found that the transcript abundance and activity level of the fatty acid β -oxidation enzyme HADHA was lower in oocytes of sedentary HFD mice than in all other groups of mice. Additionally, HFD sedentary mice had a significant increase in TCA cycle enzyme Idh3, and transcript levels of Aco2 were increased, but the difference was not statistically significant. However, exercise exposure significantly

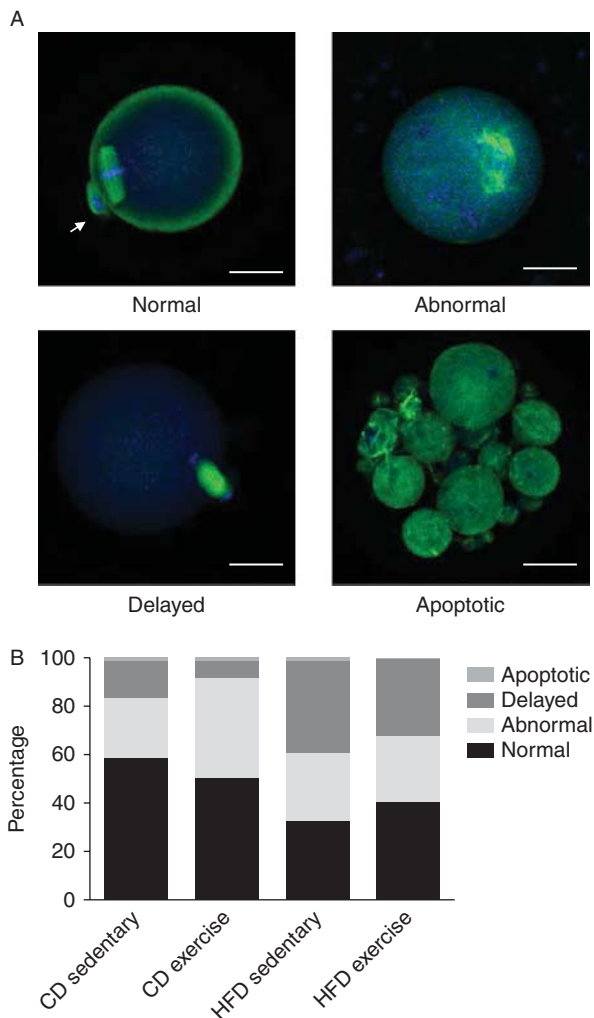


Figure 5 Meiotic progression and spindle structure of metaphase II (MII) stage oocytes. (A) Representative images of each classification category of meiotic anomalies. Arrow indicates polar body. Apoptotic indicates and apoptotic oocyte contained within the zona pellucida, scale bars: 25 μm. (B) Graph depicting percentage of oocyte in each category from each of the four experimental conditions.

increased HADHA activity and decreased *Idh3* gene expression changes in oocytes from both the HFD mice. One explanation for the increase in TCA cycle gene expression and decrease fatty acid oxidation gene expression genes is that there is chronic utilization of the TCA cycle due to the high glucose levels in the blood. In a study of patients with non-alcoholic fatty liver disease, investigators showed that there is a chronic utilization of the TCA cycle due to increased blood glucose, which results in no utilization of lipid and causes the triglycerides to accumulate in hepatocytes (Sunny *et al.* 2011). Our qPCR, metabolite and HADHA activity assays, in combination with the rescue of the HFD sedentary increase lipid, suggests a similar mechanism could be occurring in the oocytes. However,

further functional assays not conducted here must be conducted to confirm this.

This finding is in agreement with previous demonstrations that exercise increases fatty acid oxidation in mitochondria of other tissue types (Houten & Wanders 2010). Additionally, mitochondrial biogenesis is stimulated in the skeletal muscle of exercised animals (Russell *et al.* 2014), but we have not explored that possibility here.

Fatty acid β-oxidation is necessary for proper oocyte maturation and ovulation and embryo development (Dunning *et al.* 2010). For example, inhibition of β-oxidation in cumulus-enclosed oocytes prevents the oocytes from eliciting the appropriate hormone-induced meiotic activation and maturation *in vitro* (Valsangkar & Downs 2013). Additionally, defects in β-oxidation cause fertility defects in mice and humans (Dunning *et al.* 2014a). Finally, enhancing fatty acid oxidation by exposure of oocytes to L-carnitine improves the blastocyst development rate after fertilization (Paczkowski *et al.* 2014). Thus, an important question for the future is whether or not the increase in fatty acid oxidation activity induced by exercise can improve fertility.

The increased HADHA activity correlated with improvements to two aspects of mitochondrial ultrastructure: fewer rose petal mitochondria and fewer elliptical mitochondria. In oocytes, mitochondrial ultrastructure is generally round with cristae that wrap the periphery (Sathanathan & Trounson 2000). In other tissue types, particularly skeletal muscle, both exercise training and IR cause changes to mitochondrial ultrastructure and activity (Yan *et al.* 2012, Russell *et al.* 2014). Here, our data indicate that exercise may be able to induce changes to mitochondrial morphology independent of IR. HOMA-IR was unaffected by moderate exercise, yet exercise reduced the prevalence of abnormal elliptical shaped mitochondria by EM analysis. A direct relationship between glucose tolerance, IR, and mitochondrial shape was not addressed here. Still, previous research suggests impaired glucose tolerance, and insulin responsiveness can affect mitochondrial morphology (Wang *et al.* 2009, 2012, Luzzo *et al.* 2012). While the mechanism underlying the changes in mitochondrial morphology remains to be determined, we demonstrate here that exercise is clearly beneficial and may be able to override the previously observed negative effects of IR on oocyte mitochondrial morphology (Wang *et al.* 2009).

Despite the benefits of moderate exercise on lipid accumulation and mitochondrial structure in the oocytes of HFD mice, exercise did not change the proportion of oocytes ovulated with deformed spindles. One possibility is that glucose tolerance is not completely normalized by exercise, and IR is unchanged by exercise. Glucose intolerance due to IR could lead to decreased glucose uptake within the COCs during maturation. Previously, it has been shown glucose uptake by COCs is an insulin-stimulated response,

meaning it is less sensitive in situations in cases of IR and decreased glucose tolerance (Purcell *et al.* 2012). Additionally, IR and glucose intolerance in mice attenuates insulin-stimulated glucose uptake in oocytes (Wang *et al.* 2012). Importantly, the decreased glucose uptake correlated with deformed spindles. Therefore, a possible explanation for unchanged spindle morphology is persistent impaired glucose tolerance and IR in HFD mice after exercise exposure.

In conclusion, we showed that exercise was able to improve some aspects of oocyte quality in HFD-exposed mice. Whether these improvements to oocyte quality are beneficial to fertility remains to be investigated. Likewise, it will be important to determine whether exercise can improve oocyte quality in obese women who are attempting to conceive.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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