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# Chronic Supplementation with L-Isoleucine Alone or in Combination with Exercise Reduces Hepatic Cholesterol Levels with No Effect on Serum Glucose, Insulin, or Lipids in Rats Fed a High Fructose Diet

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## Abstract

The thought of using branched-chain amino acids (BCAA) in the prevention and treatment of certain disorders is becoming increasingly popular. Individual BCAA use has been associated with improving glucose tolerance and liver disease. Previous studies have cited improvements in glucose metabolism with a single dose of L-isoleucine (ILE). However, it is still unclear whether chronic consumption of ILE has any direct benefit. The objective of this study was to examine the influence of chronic ILE supplementation alone or in combination with exercise on fasting serum glucose, insulin, lipids, and lipoprotein cholesterol levels; glucose tolerance; and hepatic lipids in rats. Male Sprague-Dawley rats (n = 40) were divided into Control (low fructose diet); High Fructose diet (HF); HF plus 1.5% ILE (HF + ILE); HF plus exercise (HF + EX); and HF plus 1.5% ILE and exercise (HF + ILE + EX). The HF diets consisted of 70% kcalories from fructose. After 6 weeks of treatment, no significant differences were observed between groups for changes in fasting serum glucose, insulin, lipids, or lipoprotein cholesterol levels. However, hepatic total cholesterol was significantly lower in the HF + ILE + EX compared to the Control and HF, while, the HF + ILE had significantly lower hepatic free cholesterol compared to the HF. We also found no differences between groups for serum glucose response following an oral glucose tolerance test. In conclusion, our study shows that ILE supplementation in rats does not influence serum glucose and lipid biomarkers but may have an influence on lipid metabolic pathways within the liver.

**Keywords**

Isoleucine, Branched-Chain Amino Acids, Glucose Tolerance, Insulin, Cholesterol, Lipids

## 1. Introduction

With the rising rates of metabolic diseases and type 2 diabetes, nutritional interventions to help improve health have become increasingly important. The Branched Chain Amino Acids (BCAAs): leucine, isoleucine, and valine; are essential for healthy tissue growth and development. However, the role of BCAAs remains unclear with regards to prevention and/or treatment of metabolic diseases. Mangge *et al.* [1] reported a significant correlation beteween serum BCAAs and increased cardiometabolic risk. Contrary to this, Jennings *et al.* [2] found, in a study of twins, that participants with a higher dietary intake of BCAAs resulted in a lower prevalence of cardiometabolic risk factors, including insulin resistance.

BCAAs are commonly supplemented to stimulate muscle protein synthesis and recovery following bouts of exercise [3] [4] [5]. BCAA supplementation during endurance exercise has been shown to decrease the rate of protein degradation and delay muscle glycogen depletion [6] [7]. In regards to tissue growth, leucine, a specific branched-chain amino acid, has been shown to be a key regulator and potent stimulator of the muscle protein synthesis pathway via mTOR activation [5] [8] [9]. When examining the BCAAs individually, leucine has been shown to have a more significant impact on stimulating muscle protein synthesis, while isoleucine appears to have a greater effect on blood glucose homeostasis [10] [11] [12] [13].

Unlike leucine, isoleucine does not appear to play a pivotal role in stimulating muscle protein synthesis [10]. Instead, isoleucine's physiological impact appears to be on glucose metabolism [12]. Using rats, Doi *et al.* [10] compared leucine and isoleucine and showed that 0.45 g/kg of isoleucine significantly decreased plasma glucose compared to controls, while leucine showed no significant effect. Also, the addition of isoleucine to the diet increased skeletal muscle glucose uptake as well [10]. Another study [12] showed that isoleucine supplementation in rodents decreases the glucose response to an oral glucose tolerance test when compared to leucine and valine.

When myotubes are treated with rapamycin, an mTOR inhibitor; glucose uptake with isoleucine was significantly increased compared to the control [11]. Alternatively, when cells treated with a PI3-K inhibitor significantly decreased glucose uptake compared to the controls; thus isoleucine may influence glucose uptake through PI3-K [11]. This also suggests that in a typical BCAA supplement, the presence of leucine, which stimulates mTOR activity, may also prevent muscle tissue from reaching maximum glucose uptake.

The current state of research on isoleucine suggests that it can have a significant blood glucose-lowering effect and the use of isoleucine could play a role in

the prevention or treatment of pathologies related to glucose metabolism, such as diabetes and metabolic disease. The primary objective of this study was to examine the influence of chronic L-isoleucine supplementation on serum glucose, lipids, and lipoprotein cholesterol levels and hepatic cholesterol levels in rats fed a high fructose diet and whether exercise also impacts the effects of isoleucine on these parameters.

## 2. Materials and Methods

### 2.1. Animals and Diet

We obtained forty, male Sprague Dawley rats (weighing 350 ± 12.2 g) from Charles River Breeding Labs (Wilmington, MA). They were group housed (2 per cage) in polystyrene cages with bedding in a temperature-controlled room at 23˚C on a 12-hour light-and-dark cycle. We provided animals with tap water and food ad libitum. The animals were maintained in accordance with the guidelines set by the Committee on Animals of the University of Massachusetts Lowell Research Foundation and the National Research Councils, Committee on Care in Use of Animals Resources.

During the assimilation week, animals were fed a standard chow-based diet (Purina, St. Louis, MO). Following the one-week acclimation period we placed them on one of three semi-purified diets (Research Diets, Huntley, NJ). A control diet with no added fructose or isoleucine (Control), a high fructose diet containing 10% kcal from fat and 70% kcal from fructose (HF), and a HF plus ILE diet (which contained 10% kcal from fat, 70% kcal from fructose and 1.5% added isoleucine) (HF + ILE).

### 2.2. Experimental Groups

We randomly divided the forty animals into five equal groups of eight animals. Group 1 was fed the Control. Group 2 was HF diet. Group 3 was fed the HF + ILE diet. Group 4 was fed the HF diet and were exercised (HF + EX). Group 5 was fed the HF + ILE and exercised (HF + ILE + EX).

### 2.3. Training Protocol

We acclimated animals from both exercise groups to a rodent treadmill (Columbus Instruments, Columbus, OH) for one week. Exposure began with free roaming on an unmoving treadmill belt and gradually increased to a pace of 10 m/min for 10 minutes over the duration of 1 week. Upon initiation of the dietary intervention both exercise groups were run on the treadmill three days per week for six weeks. The animals ran for: week 1, 15 minutes at 10 m/min; week 2, 20 minutes at 15 m/min; week 3, 20 minutes at 20 m/min; week 4, 20 minutes at 25 m/min; week 5, 20 minutes at 30 m/min; and week 6, 20 minutes at 35 m/min.

### 2.4. Sample Collection

We collected blood samples for the measurements of the serum concentrations

of glucose, total cholesterol, lipoprotein cholesterol, and triglycerides following a 12-hour fasting period. We drew up to 1mL of blood from the tail vein at baseline, two, and six-week time points. Samples were centrifuged upon collection at 1500 × g 4˚C for 20 minutes and stored at −80˚C until analyses of all samples at the same time.

### 2.5. Oral Glucose Tolerance Test

At the end of 6 weeks, all animals were fasted 12 hours before the start of the oral glucose tolerance test (OGTT). Animals had a fasting blood sample collected via the tail vein and were then given an oral bolus of glucose (1 g/kg body weight) via oral gavage. Whole blood samples were collected from the tail vein at 30, 60, 90, and 120 minutes and were measured for glucose.

### 2.6. Tissue Collection and Hepatic Cholesterol Measurement

Upon completing the study, liver tissue was removed, cleaned and blotted dry before weighing and stored at −80˚C until analyses. Hepatic cholesterol concentrations were measured using a previously described method [14] [15], in the following manner: a 100 mg portion of liver was homogenized with 50 mg of sodium sulfate. Five mL of methanol was added, and the tissue homogenized a second time followed by addition of 10 mL of chloroform. After mixing, a 3 mL solution containing 1.25% KCl and 0.05% $H_2SO_4$ was added and centrifuged at 400 × g at room temperature for 10 min. The bottom layer was transferred and the supernatant was re-extracted with 3 mL of chloroform/methanol (2:1) and centrifuged at 400 × g at room temperature for 10 min. The bottom layer was transferred and pooled with the previous step. The solution was placed in a 37˚C water-bath and placed under $N_2$. When approximately half of the solution was evaporated, 1mL of chloroform with 1% Triton-100 was added, mixed and evaporated to dryness at 37˚C under $N_2$. A total of 500 mL of distilled water was added to the samples, vortexed and placed in a shaking water bath at 37˚C for 20 min to solubilize the lipid. After incubation, hepatic total and free cholesterol concentrations were determined enzymatically (Wako Chemicals, Richmond, VA). Hepatic cholesteryl ester concentration was defined as the difference between the total and the free cholesterol concentrations.

### 2.7. Serum Glucose, Insulin, Lipid and Lipoprotein Cholesterol Measurements

Serum glucose, lipids, and lipoprotein cholesterol were measured using the Medica EasyRA Clinical Chemistry Analyzer (Medica Corporation, Bedford, MA). Glucose values were calculated through an enzymatic reaction that causes the complete oxidation of glucose and the simultaneous reduction of NAD to NADH. Serum triglycerides values were calculated based on the reaction rate of a catalytic enzyme. Serum total cholesterol was quantified using an enzymatic Trinder endpoint reaction. Serum high-density lipoprotein (HDL) cholesterol quantification required two reagents. The first reagent removed the non-HDL compo-

nent of the lipoproteins in the sample, and the second reagent acted as a detergent to solubilize HDL. Serum low-density lipoprotein (LDL) cholesterol reacted with a reagent to solubilize the non-LDL particles. The cholesterol realized was then consumed by cholesterol esterase and cholesterol oxidase in a non-color forming reaction.

Serum insulin was measured using the Millipore sandwich type immunoassay for Rat/Mouse Insulin ELISA kit (EMD Millipore Corporation, Billerica, MA). The 96-well microplate was washed with Wash Buffer using an automated washer (BioTek Instruments, Winooski, VT, USA). Following the 3-washes of the plate the standards, controls, and samples were added to the microplate wells in duplicate. Detection Antibody was then added to all wells and the microplate was then incubated on a microplate shaker at 500 rpm for two hours. After the first incubation was complete, the solutions were decanted, and the plate was washed. The enzyme solution was added to each well and incubated for 30 minutes followed by decanting and washing. A substrate was added to each well and incubated on a shaker for 20 minutes. Stop Solution was then added to each well and the absorbance at 450 nm, and 590 nm was quickly measured by the Tecan Infinite 200 Pro microplate reader (Tecan Group Ltd., Mannedorf, Switzerland). The intensity of the color generated was directly proportional to the amount of insulin in the sample.

### 2.8. Statistical Analyses

Statistical evaluations were performed using SPSS for Mac 24.0 software (SPSS, INC., Chicago, IL). The dataset was analyzed using a one-way ANOVA, two-way mixed ANOVA, and t-tests where appropriate to assess within group and between-group variations. Post hoc analysis was performed using Tukey's test when appropriate. We calculated area under the curve (AUC) for the glucose tolerance test. All values are expressed as means ± SD and statistical significance was set to a $p$-value ≤ 0.05.

## 3. Results

### 3.1. Animal Characteristics

A total of 40 Sprague-Dawley rats, randomly placed into five groups of eight completed the study. The average baseline weight was 353.65 ± 12.28 g with no significant difference between groups. Following the six-week diet and exercise intervention, the average weight gain was 137.73 ± 46.92 g. The Control group gained the most weight, with an average increase of 170.12 ± 33.92 g, whereas, the HF + EX group gained the least with an average increase of 113.50 ± 38.78 g. All groups gained a significant amount of weight when comparing their baseline and final weights at 6 weeks ($p \leq 0.05$). Between groups, the HF + EX group had a significantly lower weight at every time point when compared to the Control group ($p \leq 0.05$). The HF group had a significantly lower weight than the control at weeks one, three, five and six ($p \leq 0.05$). HF + EX + ILE weighed significantly

less than the Control group at week 4 ($p \leq 0.05$) (Figure 1).

When we calculate the average rate of weight change ($\frac{\Delta y}{\Delta x}$, where $y$ = weight and $x$ = time) we find the HF group and the HF + EX group both gained at a significantly slower rate than Control group ($p \leq 0.05$). The Control group gained weight at an average rate of 28.35 g/week whereas the HF group gained at 22.94 g/week and the HF + EX group gained 18.92 g/week ($p \leq 0.01$).

### 3.2. Dietary Intake

No significant difference was seen between the groups for total food intake at any time point (data not shown). On average, each animal consumed approximately 21.20 ± 2.30 g/d. This equated to an average intake of 81.53 ± 8.85 kCal/d. Animals in the Control group consumed a weekly average of 4.42 g leucine, 2.46 g ILE, and 3.03 g of valine. Animals in the HF and HF + EX consumed an average of 4.52 g leucine, 2.38 g ILE, and 2.93 g of valine. Animals in the HF + ILE and HF + ILE + EX consumed 55% more ILE than the other groups ($p \leq 0.005$). On average, animals within in the ILE groups consumed 6.9 grams of ILE each week, 4.42 g of leucine, and 2.96 g of valine.

### 3.3. Fasting Serum Glucose and Insulin, Lipid and Lipoprotein Cholesterol Measurements

Serum glucose concentrations from baseline to six-weeks within each group were not significantly different from each other (Table 1). Although insignificant, the

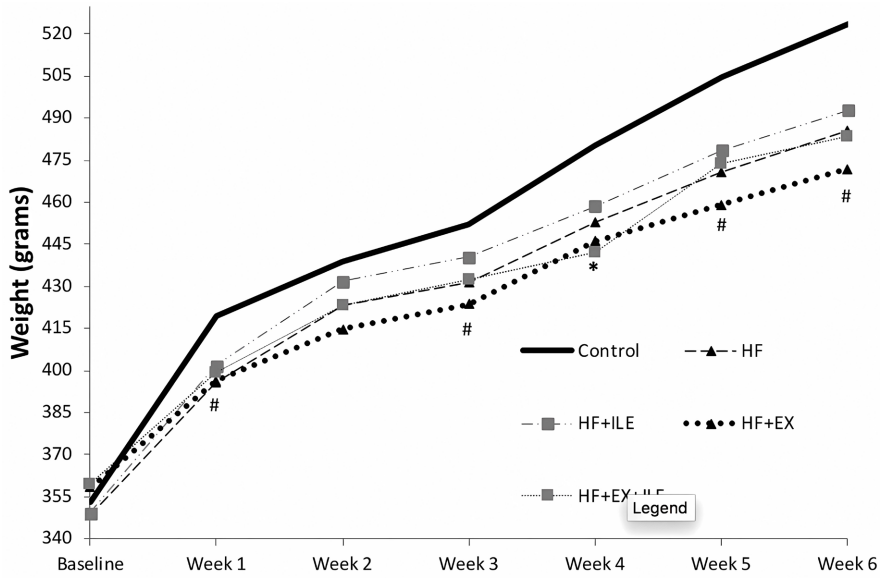


Figure 1. Sprague-Dawley rats (n = 40; 8/group). Average weight from baseline through the end of the 6th week. Each group gained a significant amount of weight when comparing their baseline to final ($p < 0.05$). HF had significantly lower body weight at weeks one, three, five and six (#, $p < 0.05$). The HF + EX group had a significantly lower weight than the Control group at every time point ($p < 0.05$). The HF + EX + ILE group weighed significantly less than the Control at week four (*, $p < 0.05$).

**Table 1.** Fasting serum glucose, insulin, lipids and lipoprotein cholesterol concentrations at baseline and after 6 weeks of intervention. Values displayed as group mean (standard deviation). * Significant difference from baseline, $p < 0.05$.

| | Control | | HF | | HF + ILE | | HF + EX | | HF + ILE + EX | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Baseline | Final | Baseline | Final | Baseline | Final | Baseline | Final | Baseline | Final |
| Glucose (mg/dL) | 110.50 (23.08) | 120.88 (21.63) | 103.13 (29.47) | 120.62 (19.69) | 110.25 (26.30) | 136.50 (44.43) | 100.37 (17.21) | 112.12 (34.03) | 131.62 (21.39) | 126.37 (26.31) |
| Insulin (pg/mL) | 247.50 (93.62) | 422.25 (421.07) | 200.00 (144.22) | 448.75 (388.93) | 330.00 (231.21) | 355.00 (385) | 200.00 (140.61) | 135.00 (43.35) | 306.25 (316.90) | 187.50 (179.02) |
| Total Cholesterol (mg/dL) | 73.38 (11.25) | 78.38 (14.60) | 80.25 (14.68) | 79.28 (10.93) | 76.12 (12.03) | 86.12 (18.12) | 80.50 (15.81) | 81.12 (18.89) | 73.75 (14.22) | 80.12 (14.62) |
| HDL-cholesterol (mg/dL) | 24.63 (2.45) | 24.75 (4.40) | 24.86 (3.98) | 27.25 (2.92) | 25.12 (2.95) | 26.87 (5.00) | 26.75 (3.41) | 26.62 (3.66) | 27.00 (4.41) | 25.62 (4.03) |
| LDL-cholesterol (mg/dL) | 13.13 (4.49) | 10.43 (2.37) | 13.37 (2.83) | 11.00 (1.20) | 11.62 (3.02) | 11.5 (4.21) | 14.62 (4.50) | 12.25 (4.53) | 11.75 (3.24) | 11.00 (3.38) |
| Triglycerides (mg/dL) | 53.25 (19.24) | 159.63* (43.32) | 48.37 (20.33) | 160.37* (59.75) | 51.50 (12.01) | 165.62* (72.67) | 43.88 (10.41) | 125.87* (44.64) | 61.50 (17.86) | 125.88* (36.49) |

HF + ILE group had slightly higher values after 6 weeks of treatment compared to non-ILE treatment groups and the two EX groups (HF + EX and HF + EX + ILE) were slightly lower than their respective non-EX groups (HF and HF + ILE) (Table 1).

Serum insulin concentrations were not significantly different between groups at any time point (Table 1). However, the two exercise treatment groups had slightly but not significantly, lower serum insulin concentrations after 6 weeks of treatment (HF + EX, −32.50% and HF + ILE + EX, −38.77%) while the Control group (70.70%) and the HF group (124.37%) increased (Table 1) from baseline to 6 weeks.

Serum total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglyceride concentrations were not significantly different between treatment groups at either time point (Table 1). However, serum triglyceride concentrations did show a significant increase ($p \leq 0.05$) across all treatment groups, including the Control, from baseline to 6 weeks (Table 1). The Control group increased 199.76%, the HF group increased 231.55%, the HF + ILE group increased 221.59%, the HF + EX group increased 141.88%, and the HF + ILE + EX group increased 104.68% (Table 1).

### 3.4. Hepatic Cholesterol Levels

We found the HF + ILE + EX group had a 131.86% lower total hepatic cholesterol than the Control, and 113.73% less than the HF group ($p \leq 0.05$) (Figure 2). Free cholesterol was found to be significantly lower in the HF + ILE group when compared to the HF group (145.31% decrease, $p \leq 0.05$). Hepatic Cholesterol Ester was not found to have any significant differences between groups (Figure 2).

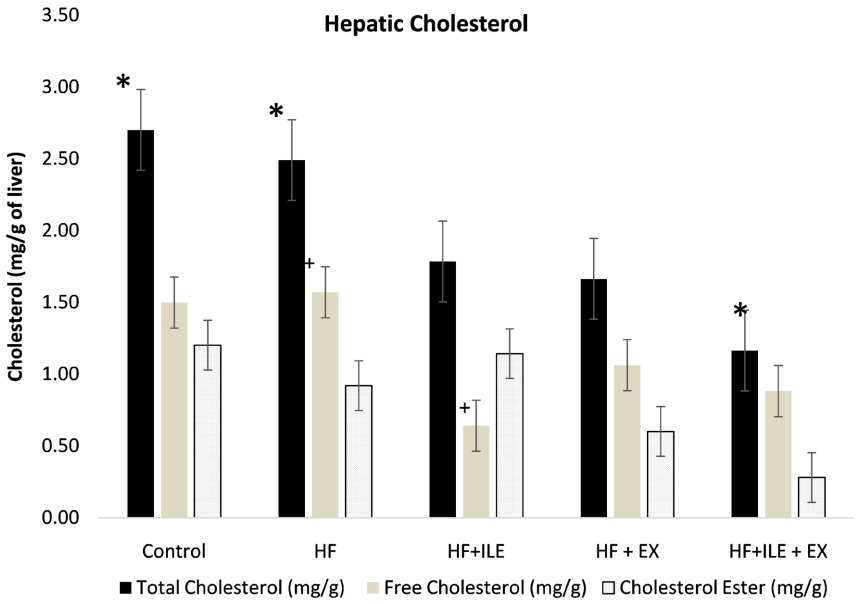


**Figure 2.** Hepatic Cholesterol (mg/g liver) following six weeks of treatment. HF+ILE+EX group had a significantly lower total cholesterol level than the Control and HF group (*, $p<0.05$). Free cholesterol was found to be significantly lower in the HF+ILE group when compared to the HF group (+, $p<0.05$). Hepatic Cholesterol Ester was not found to have any significant differences between groups.

### 3.5. Oral Glucose Tolerance Test

With the exception of HF group, each group had a significant increase in serum glucose from baseline to 30 minutes ($p \leq 0.05$) following a single oral dose of glucose (Table 2). At 60-minutes every group except the HF + EX was significantly elevated from their baseline measurement ($p \leq 0.05$). At 60-minutes the HF group was the only group continuing to rise; all other groups began returning towards baseline values (Table 2). At 90 minutes the Control, HF, and HF + ILE + EX groups remained significantly elevated from baseline ($p \leq 0.05$). At 120 minutes, the Control group remained 14.09% elevated from baseline ($p \leq 0.05$), HF was elevated 10.71%, and HF + ILE was elevated 8.21%. The HF + EX group was the only group to drop below baseline with a 5.05% decrease from baseline. HF + ILE + EX remained slightly elevated from baseline with a 2.98% increase. Between groups we see significantly lower blood glucose in the HF + EX group at 90-minutes when compared to HF + ILE ($p \leq 0.05$). A significant decrease in blood sugar was seen at 120 minutes in the HF + ILE + EX group when compared to the Control and HF + ILE groups ($p \leq 0.05$). When we looked at the total response and calculated the Area under Curve (AUC), we noticed the HF (12961.88) and HF + ILE (12941.25) had nearly identical responses. Exercise groups had a slightly reduced AUC compared to the Control and non-exercise groups. We found no significant differences between treatments for AUC (Table 2).

## 4. Discussion

As expected with the normal growth of Sprague-Dawley rats, the average weights

**Table 2.** Blood glucose response to oral glucose tolerance test following six weeks of treatment. Significant elevations were seen within each group 30-minutes and 60 minutes after feeding ($p < 0.05$).

| | Time | | | | | AUC |
|---|---|---|---|---|---|---|
| | Baseline | 30 minutes | 60 minutes | 90 minutes | 120 minutes | |
| Control | 93.13 (8.11) | 121.50[a] (22.49) | 114.75[a] (13.52) | 106.88[a] (13.27) | 106.25[a] (13.86) | 13254.38 (1512.77) |
| HF | 94.50 (7.03) | 112.13 (19.74) | 116.13[a] (15.32) | 104.25[a] (7.65) | 104.63 (11.96) | 12961.88 (991.78) |
| HF + ILE | 98.75 (13.16) | 116.13[a] (13.62) | 112.38[a] (13.46) | 106.75[c] (9.18) | 106.86 (12.68) | 12941.25 (1384.06) |
| HF + EX | 98.25 (15.40) | 112.50[a] (10.53) | 106.75 (11.48) | 95.12 (11.48) | 93.29 (11.38) | 12129.38 (1086.88) |
| HF + ILE + EX | 88.00 (5.29) | 111.75[a] (11.73) | 109.88[a] (16.15) | 101.50[a] (9.78) | 90.63[b] (6.39) | 12373.13 (654.98) |

Values displayed as mean (standard deviation), *a:* significant difference from baseline; *b:* significantly different from control; *c:* significantly different than HF + EX; *d:* significantly different than HF + ILE ($p < 0.05$).

increased throughout the study and each group significantly increased weight from baseline to final. Within each group, the average increase was 41% over the six-week period. The HF + EX group had a significantly lower weight than the control at every time point and the HF + EX + ILE group had significantly lower weight at week four ($p \leq 0.05$). No significant differences occurred in the total food consumption, which was on average a total of 1141 kCals of food per week or an average of 296.83 grams per week (148 grams per animal, per week). Amino Acid consumption showed no significant difference between the control, HF, and HF + EX groups. The HF + ILE and HF + EX + ILE groups consumed an average of 187% more ILE than the non-ILE groups. The increase in ILE did not lead to any significant changes in weight gain. It appears that while exercise was able to attenuate the gain in weight, ILE did not influence the change in weight at the current dosage level.

Previous studies have shown that blood glucose is lowered in rodents when supplemented with ILE. The glucose-lowering is associated with increased muscle glucose uptake along with no significant changes in serum insulin [10] [11]. Prior studies have primarily assessed the impact of ILE through a glucose challenge with or without ILE. In our design, we provided rats with either a traditional chow or one enriched with ILE. We found that the addition of ILE to every meal caused no significant changes in fasting blood glucose or insulin, although there were some trends on positive changes with serum insulin levels, which may have been significant if not for the great variability in mean levels between groups baseline. The possible reason for this variability is unknown. Contrary to our results, a previous study in glucose intolerant mice showed a slight, but significant, decrease in fasting blood glucose. In their study, ILE was

provided *ad libitum* at a 2% concentration of the water source [13] rather than through the diet as in the current study.

In the present study we examined how treadmill running would influence the effects of ILE. While we did not identify a statistically significant difference we did see a trend towards a lower fasting glucose. Following the six-week treatment the HF + ILE + EX group was the only group to have a fasting glucose measurement less than the baseline measurement. HF + ILE + EX reduced glucose by 3.99%, whereas the HF + EX group increased 11.71%. We also saw the HF + ILE + EX group reduce fasting insulin 38.75% and the HF + EX group decrease 32.5%. Although not significant in this study it doses suggests there may be additive effect on glucose management when ILE and exercise are combined and could be an area for future study.

To our knowledge, this is the first study to specifically look at the influence of ILE in isolation on lipids. Total cholesterol did not significantly vary between treatments and remained relatively unchanged. ILE groups had the most substantial increases in cholesterol, but the elevation did not demonstrate a significant increase. Exercise groups also had no significant difference in total cholesterol levels. HDL remained relatively unchanged from baseline to final in all groups, and LDL decreased slightly in all groups except HF + ILE. Overall, total, HDL, and LDL cholesterol remained unchanged over the course of the six-week treatment. Triglyceride values increased significantly from baseline to final in all groups. Exercise did attenuate the elevation in triglycerides but did not significantly reduce values when compared to the non-exercise groups. While not significant, the smallest increase in TG levels was found in the HF + LE + EX group. With the high fructose diet, we anticipated an elevation in triglyceride levels. Previous studies have demonstrated an increase in blood triglyceride levels with a high fructose diet [16] [17].

Previous rodent studies have demonstrated a significant decrease in the blood glucose response following an oral glucose tolerance test when ILE is provided [10] [12] [18]. The majority of previous work had specifically looked at the acute change in the glucose response when the OGTT was supplemented with ILE. In the current study, we performed the OGTT without ILE to determine if chronic feeding would alter the glucose response in the absence of ILE. We showed that providing ILE at every meal in a concentration 1.5 times greater than Leucine, and 2.3 times greater than Valine, did not influence glucose regulation after six weeks. This suggests that ILE must be supplemented during a high glucose meal to see benefits. Neither of the ILE groups showed a significant improvement in glucose AUC. To our knowledge, this is the first rat study to measure glucose response to a traditional OGTT following chronic feeding of ILE. Our findings align with the previously referenced study in mice by Ikehara *et al.* [13]. Both studies found no change in glucose response when ILE was not provided during the OGTT. While we did not measure the insulin response to the OGTT, Ikehara *et al.*, noted significantly lower insulin AUC, and suggested that role of ILE in

glucose regulation may be through an insulin-independent mechanism [13].

We found that six weeks of supplementing ILE to meals does not significantly impact glucose or blood lipid profiles. We also found no significant influence of exercise combined with ILE on blood profiles. In the current study animals fed the ILE consumed an average of 0.97 g/day per cage, for an estimated 0.48 grams per animal each day. Previous work had identified 0.45 g/kg to be the optimal quantity of ILE to produce glucose lower effects [10]. Our study introduced chow fortified with ILE and greatly reduced the g/kg consumed. We have a second significant study design variation, wherein we provided the ILE throughout the 24 hours eating cycle and not in a single dosage. The dosage of ILE per meal may have been too low to alter glucose metabolism in the current study compared to previous studies showing a lowering in blood glucose.

Our analysis of hepatic cholesterol showed that ILE had a positive influence on hepatic cholesterol. Total hepatic cholesterol decreased 131.86% with HF + ILE + EX compared to the control, and 113.73% compared to HF ($p \leq 0.05$). This decrease was also significant with free cholesterol when comparing HF vs. HF + ILE (−145.31%) ($p \leq 0.05$) (**Figure 2**). This is the first study we are aware of to identify changes in hepatic cholesterol with ILE feeding. Previously it had been identified that ILE decreases hepatic gluconeogenesis in Wistar rats [10]. The reduction of hepatic gluconeogenesis is one mechanism proposed to explain the glucose lower effects of ILE.

Multiple research groups have a looked at the impact of BCAAs on symptoms and progression of liver disease. BCAA supplementation has been shown to improve the outcomes of patients with liver disease [19] [20]. In an analysis of hepatic cholesterol in patients diagnosed with either non-alcoholic fatty liver (NAFL) disease or non-alcoholic steatohepatitis (NASH), an increase in free cholesterol is seen without an associated increase in cholesterol esters [21]. Much like the research on BCAAs in exercise, the research looking at the influence on liver disease has primarily looked at BCAAs as a group. Future studies should further examine how ILE alone may positively influence liver metabolism of lipids and glucose.

## 5. Conclusion

We demonstrated that the addition of 0.48 g/day of ILE for six weeks did not alter glucose metabolism compared to animals not consuming ILE. Furthermore, we also did not find an additive effect of ILE plus exercise on glucose metabolism. However, the current study did show a positive influence of ILE on hepatic cholesterol levels. Further research needs to be completed to identify how ILE alone may be used to benefit patients with liver disease and associated symptoms such as insulin resistance.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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## List of Abbreviations

BCAA (Branched chain amino acids);
mTOR (mammalian target of rapamycin);
PI3-K (Phosphoinositide 3-kinase);
ILE (Isoleucine);
HF (High fructose diet);
HF + ILE (High fructose diet with isoleucine);
HF + EX (High fructose diet plus exercise);
HF + ILE + EX (High fructose diet with isoleucine and exercise);
EX (Exercise);
OGTT (Oral glucose tolerance test);
KCL (Potassium chloride);
$H_2SO_4$ (Sulfuric acid);
$N_2$ (Nitrogen gas);
HDL (High density lipoprotein);
LDL (Low density lipoprotein);
ELISA (Enzyme-linked immunosorbent assay);
AUC (Area under curve);
NAFL (Non-alcoholic fatty liver);
NASH (Non-alcoholic steatohepatitis).