Indole-3 carbinol on lipid accumulation in *Caenorhabditis elegans* as a novel therapeutic for Type II Diabetes

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SUMMARY

Over the past 35 years, an epidemic in diabetes has caused a spike in health and cardiovascular complications worldwide, impacting especially our most vulnerable populations. An accumulation of lipids in cells plays an important role in the development of Type 2 Diabetes (T2D), a chronic condition that affects the way the body processes blood sugar. The pathology of T2D is marked by an increased deposition of lipids with subsequent decreases in insulin sensitivity. This lipid-induced hepatic insulin resistance, however, is difficult to target because the molecular mechanisms regarding the pathogenesis of T2D have remained elusive. Despite this, there is reason to believe that Indole-3 carbinol (I3C), which regulates the expression and activity of enzymes involved in the metabolism and elimination process of organic compounds, may therefore provide a valuable therapeutic strategy against the lipid accumulation associated with T2D. This study investigates the effect of the antioxidant I3C as a prospective novel treatment to reduce lipid accumulation, thus countering the progression of T2D. The purpose of this study was to test if exposing a model organism to varying amounts of I3C would lead to a decrease in lipid levels. Using transgenic *Caenorhabditis elegans* (Strain LIU1) to model T2D, this study found a correlation between an I3C high diet and decreased lipid accumulation, suggesting that it may be an effective treatment.

INTRODUCTION

Diabetes is a chronic disease that influences the way the body processes blood sugar, such that there is not enough insulin available to regulate the uptake of glucose from the blood (1). Diabetes affects just over 1 in 10 individuals in the United States, totaling around 34 million Americans (2). The pancreatic islets, a group of cells of the pancreas, produce β-cells that release the hormone insulin to regulate blood sugar levels by promoting glucose uptake into the muscles, liver, and adipose (fatty) tissue (3). In Type 2 Diabetes (T2D), lipid-induced insulin resistance, a decreased sensitivity to insulin in normally-insulin responsive tissues, leads to large amounts of glucose staying in the bloodstream rather than being properly absorbed (4). To counteract the high amount of glucose in the blood, the pancreas increases the secretion of insulin, which eventually leads to β-cell dysfunction (5). High amounts of insulin for a prolonged period of time promote

lipogenesis, the creation and storing of fat, and prevent lipolysis, the breakdown of fat (6). The result is an increased import and storage of these molecules in non-adipose tissues; this ectopic storage of lipids in the cells can develop into lipid-induced toxicity (lipotoxicity) that further increases systemic insulin resistance (7).

It is known and accepted that T2D is characterized by a decreased response to insulin which leads to a build-up of glucose in the bloodstream (15). However, recently there has been great controversy regarding the exact factors involved in the development of this insulin resistance (16). It is currently hypothesized that a combination of environmental factors, such as a sedentary lifestyle and a diet with high cholesterol and fat, play a key role in T2D pathogenesis, but the specific causes behind the lipid-induced insulin resistance are unknown (17). In contrast to current treatment approaches, such as insulin shots, which aim to manage the disease after the pancreas and β-cells have been affected, this study investigates a diet modification to suppress the progression of T2D before the lipotoxicity is able to cause lipid-induced insulin resistance (18).

Indole-3 Carbinol (I3C) is derived from the breakdown of glucobrassicin, a compound found in cruciferous vegetables such as broccoli and cauliflowers (8). I3C has been found to modulate the activity of enzymes involved in the metabolism and elimination of organic compounds, such as the excess lipids associated with T2D (9). Since it regulates the metabolism of these lipids, I3C has the potential to be both a potent anti-lipotoxicity agent and a valuable therapeutic strategy (10).

Across a vast variety of organisms, ranging from bacteria to humans, fats are stored in structures called lipid droplets (LDs). These fat-storing LDs contain proteins in the hydroxysteroid dehydrogenase family. A member of this protein family, dhs-3, is abundantly found in *Caenorhabditis elegans* strain LIU1 LDs (11). *C. elegans* are commonly used as a model organism in scientific research because of their short life spans and easily tractable nervous systems (12). In this study, the dhs-3 protein was used because it aggregates when there is an increase in lipids. We used a *C. elegans* line expressing GFP linked to the dhs-3 protein as a marker for lipid presence in order to visualize increased lipid levels (13). Thus, GFP aggregation, i.e., fluorescence, serves as a marker for lipid levels in this organism (14). This is significant because when there are not excess lipids present, there is more insulin sensitivity, meaning that more glucose is being taken up into the cells which would help inhibit the progression of T2D overall (**Figure 1**).

Decrease in Flourescence Intensity → Decrease in protein dhs-2 aggregation → Decrease in GFP expression → Decrease in Insulin production → Increase in insulin sensitivity

Figure 1. A schematic representation of the relationship between Fluorescence Intensity and Lipid Accumulation in C. elegans strain LIU1. The image is graphically organized to show the dhs-3 protein aggregation in relation to an increase in fluorescence in C. elegans. This format was used to show the process of how fluorescence was used as a marker for lipid presence in this study, and to clearly lay out why this transgenic line of C. elegans was used to model lipid levels in T2D.

We sought to test the hypothesis that a diet rich in I3C could lead to a decrease in lipid levels and serve as an anti-lipid-induced insulin resistance agent in response to the lipotoxicity characteristic of T2D. Based on the collected data, it was concluded that the observed decrease in lipid accumulation in the *C. elegans* model was linked to a high I3C diet.

RESULTS

To determine whether I3C treatment affected lipid accumulation, we treated the LIU1 *C. elegans* with increasing concentrations of I3C and used fluorescence microscopy to quantify levels of dhs-3-GFP accumulation. Ultimately, we used these values to calculate the Corrected Total Area Fluorescence (CTAF) values. CTAF is a measurement of fluorescence intensity calculated by subtracting the worm area multiplied by the actual mean background from the integrated density value. The actual mean background brightness was found by subtracting the given mean worm brightness from the mean background brightness. When compared to the control (water), the I3C treatment groups in low (20 µg/mL), medium (50 µg/mL), and high (81 µg/mL) concentrations exhibited significantly decreased levels of GFP fluorescence (**Figure 2**). Since we used a transgenic *C. elegans* line expressing GFP linked to the dhs-3 protein as a marker for lipid presence in order to visualize increased lipid levels such as those present in T2D, we used these results to suggest a correlation between I3C and decreased lipid accumulation in this model organism. The results of the one-way ANOVA test showed a p-value of 0.0325 (*$p<0.05$), thus showing that there was a statistically significant difference between the average CTAF values amongst the treatment groups. The Tukey HSD test determined a p-value that was in further agreement with the 0.05 *p*-value given by the ANOVA test. It is also important to mention that these findings would also suggest that the observed I3C effects are concentration-dependent, meaning that as the administered dose increases, so does the effect size. Using this information, we therefore concluded that the effects of I3C resulted in significantly lowering lipid levels of the *C. elegans* in a dose-dependent manner.

DISCUSSION

The findings suggest that I3C had significant, lipid-reducing effects in *C. elegans*. The data showed a decrease in fluorescence values, suggesting decreased lipid levels as a result of increased I3C intake. The data demonstrated that the *C. elegans* that consumed large amounts of I3C had significantly decreased fluorescence intensities when compared to those not introduced to the compound. The

results therefore supported the formulated hypothesis and were consistent with the central idea presented in literature that I3C may possess therapeutic efficacy (19).

As with any research, there were a few key limitations posed. Perhaps the largest limitation associated with this project was the stark contrast between testing a potential drug on a *C. elegans* model in comparison to the much more complicated and intricate systems of the human body involved in the development of pathologies such as T2D.

I3C instability and light sensitivity posed another key limitation. As mentioned earlier, the compound directly found in cruciferous vegetables is glucobrassicin, which is metabolized to I3C, its unstable intermediate. While I3C is a phytochemical that is derived from the breakdown of glucobrassicin, the amount of I3C metabolized from glucobrassicin is not a 1:1 ratio (20). Therefore, an individual's metabolic rate, affected by factors such as age and physical activity, may influence how much I3C is yielded from a set amount of glucobrassicin (21). Thus, it is difficult to assign a set amount of I3C to intake seeing that these factors vary so widely in the human population. Nevertheless, a range of recommended intakes can still be valuable.

Variability in *C. elegans* fluorescence and microscope setup may have also contributed as a limitation. To assure
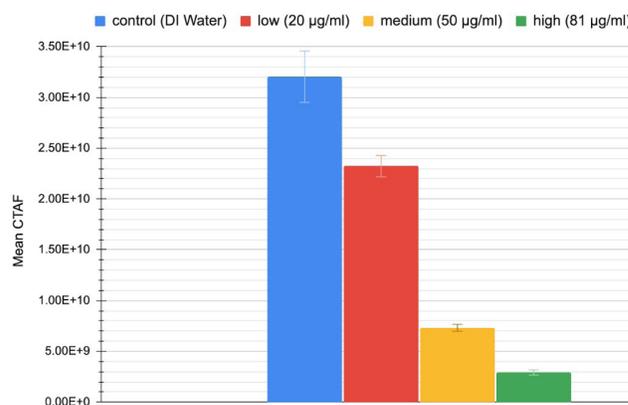
Figure 2. Average CTAF fluorescence values Average CTAF values following 24-hour exposure to I3C as an indicator for lipid reduction effects. Bar graph showing mean CTAF values of all 4 treatments (n=40). CTAF plotted on y-axis with conventional arbitrary units (a.u.). Error bars present ±2SEM. LIU1 C. elegans were grown in either control conditions, 0.8 mL DI water, or in treatment groups (low 20 µg/mL, medium 50 µg/mL, or high 81 µg/mL) for 24 hours. A p-value of less than 0.05 (*$p<0.05$), outputted from the one-way *ANOVA* analysis, is also included in the figure to indicate the statistical significance of the data.

that discrepancy in fluorescence values was due to I3C introduction only, the position of the *C. elegans* in the camera scope needed to be exactly the same for all of the images. However, this was difficult to control as the *C. elegans* were pipetted into the agar pads with sodium azide liquid to paralyze them for imaging. But this added liquid also resulted in the *C. elegans'* position not being the same for each data point, which may have contributed to slightly offsetting the outputted fluorescence values. *C. elegans* strain NC3292 may also have had an optimal fluorescence that cannot be drastically manipulated through biochemical means.

To further support these results, however, future studies conducted *in vivo* and in more complex mammalian models and environments are needed in order to make a stronger conclusion before testing I3C in clinical trials in humans. Testing the effects of I3C in organisms more closely related to humans, such as mice, that have developed T2D would also offer greater insight into its ability to promote enough lipid breakdown to counter theinsulin resistance. Further studies run in mice have shown reductions in fat accumulation which were associated with improved glucose tolerance as a result of I3C introduction, however, these mice were also subject to synthetic, very controlled diets in ways that may not attainable in humans (27). In addition, testing the prolonged effects of introducing the compound daily along with its byproducts would be needed to confirm the safety and efficacy of I3C.

In the case that I3C is further tested and its effects on β-cell growth in more complex models are confirmed, then it could be evaluated as a potential treatment option that may help counter the decreased insulin sensitivity of T2D by reducing the amount of lipid accumulation. Furthermore, a personalized diet could be made for patients with T2D so that they are intaking a large enough amount to counteract the insulin resistance. This may be especially beneficial in the case that the disease has fully developed and therefore, the body has already lost a significant amount of insulin sensitivity.

This study suggests that I3C may have an effect on lipid reduction that could be beneficial in counteracting the response of the body in T2D (**Figure 3**). However, this conclusion was based upon a limited sample size, so more data looking at its effects over extended periods of time is necessary to determine if the hypothesis proposed that I3C may decrease lipid amounts is valid. These further studies are needed on models more closely related to humans in environments less controlled than those run in this study using *C. elegans*. After testing on more complex models such as rodents, human clinical trials would be the ultimate test to confirm the efficacy and overall validity of I3C as a possible option to help counteract the pathogenesis of T2D.

MATERIALS AND METHODS
Culturing *C. elegans*

C. elegans strain LIU1 was received from the Caenorhabditis Genetics Center (22) and cultured on nematode growth medium (NGM) agar plates with *Escherichia coli* (*E. coli*) K12 as their food source (23). *C. elegans* procedures were adapted from the standard procedures described in the WormBook (24). To make 1 L of NGM Agar for making roughly 70 plates, 500 mL of deionized (DI) water and 14.58 g of NGM agar premix (Teknova) were swirled and microwaved until the mixture fully dissolved into a translucent, bright yellow color.

Five 200 mL erlenmeyer flasks were autoclaved for 1 hour. After autoclaving, 0.2 mL of 1 M magnesium sulfate ($MgSO_4$), calcium chloride ($CaCl_2$), and cholesterol supplement ($C_{27}H_{46}O$) were added to each of the five erlenmeyer flasks as an additional nutrient source. To complete the plates, 0.2 mL of *E. coli* were pipetted onto the plates after the agar plates were left to settle for 1 hour. Following *E. coli* exposure, the plates were then incubated at 37° Celsius overnight (about 12 hours).

Age Synchronizing *C.elegans*

M9 buffer was prepared by dissolving 3 g of monopotassium phosphate (KH_2PO_4), 6 g of disodium phosphate (Na_2HPO_4), and 5 g of sodium chloride (NaCl) in 1 L of DI water - all chemicals received from Sigma Aldrich. After the M9 Buffer was autoclaved for 1 hour, *C. elegans* LIU1 cultures were exposed to 2 mL of M9 buffer and swirled for 30 seconds. The liquid mixture was then pipetted into a centrifuge tube and centrifuged for 1 minute (Centrifuge settings at 2500xg, 21° Celsius, 9ACC, 9DEC, and 3659 Bucket). 20% alkaline hypochlorite solution (15mL) was poured into the centrifuge tube for 5 minutes. The alkaline hypochlorite solution was prepared by measuring 10 mL of a 5% (non-germicidal) bleach solution mixed with a 1 M sodium hydroxide (NaOH) solution made with 50 mL of DI water and 2 g of NaOH. The mixture was centrifuged again for 1 minute at the same settings, and 2 mL of M9 buffer was poured into the centrifuge tubes once more (repeated 3 times). 500 μL of the solution were then pipetted onto NGM Agar plates. The plates were placed on a *C. elegans* rocker overnight (around 12 hours), and aluminum foil was placed on top of the plates.

Introducing I3C to Cultures and Experimental Trial Set Up

I3C was introduced via a stock solution on top of the *C. elegans* NGM Agar and *E. coli* plates. Preliminary studies were conducted to identify the maximum amount of I3C that *C. elegans* could tolerate without the compound inducing their death, and it was therefore determined that the administered I3C dosage range would be 20 μg/mL – 81 μg/mL**.** To introduce *C. elegans* strain LIU1 to the compound 24 hours after age synchronization, a 1 mg/mL I3C stock solution, was made by mixing 100 mg of I3C powder (Sigma Aldrich) and 100 mL DI water on a stir plate for 1 hour. To prepare the low dosage (20 μg/mL) group, 0.8 mL of a solution of DI water with 1mL of the I3C stock solution was pipetted into an age synchronized plate. For the medium dose (50 μg/mL), 0.8 mL of a solution of DI water and 2.5 mL of the I3C stock solution were added to an age synchronized plate, and for the high concentration (81 μg/mL), 0.8 mL of a DI water and 4.05 mL I3C stock solution was introduced to the age synchronized plate. A control plate was also set up with 0.8 mL of DI water. All 4 plates were placed in a dark drawer for 24 hours prior to data collection.

Data Collection

Every trial consisted of four plates, each exposed to one of the four following conditions: Water, I3C low, I3C medium, or I3C high. Following a 24-hour exposure period to their respective experimental conditions, 10 fluorescence images were captured per plate. To reduce experimental uncertainty, four biological replicates were run. This yielded

160 total images, and therefore 40 technical replicates per experimental condition.

To collect fluorescence images of *C. elegans* strain LIU1 24 hours following I3C exposure, the micromanager software, an open-source image analysis software plug-in by Ron Vale for Image J developed by the National Institutes of Health, was used to capture the images from the AmscopeFM690T fluorescence microscope (25). *C. elegans* were placed on 2% agar pads, made with 0.4 g of Agar and 20 mL DI water microwaved and pipetted into metal washers and given 20 minutes to settle. 5 µL of a 0.7% sodium azide (NaN$_3$) solution was pipetted onto the 2% agar pads which were set on glass microscope slides. The slides were placed in a dark room and one photo was taken per individual *C. elegans* using a Point Grey GRAS-20S4M-C microscope camera. Ten fluorescence images were taken for every group, totaling 40 images per trial.

Analyzing Fluorescence Data

To determine whether I3C treatment affected lipid levels, fluorescence microscopy was used to determine levels of GFP aggregation. For fluorescence image analysis, the image FIJI distribution of ImageJ software was used. To prepare the image for analysis and enhance the contrast to make the *C. elegans* more easily distinguishable from the background, the background was subtracted. To take the measurement for *C. elegans* fluorescence intensity for each image, a polygon outlining the shape of the worm was made. In this way, ImageJ could output values including the area of the selected worm, the minimum, maximum, and mean
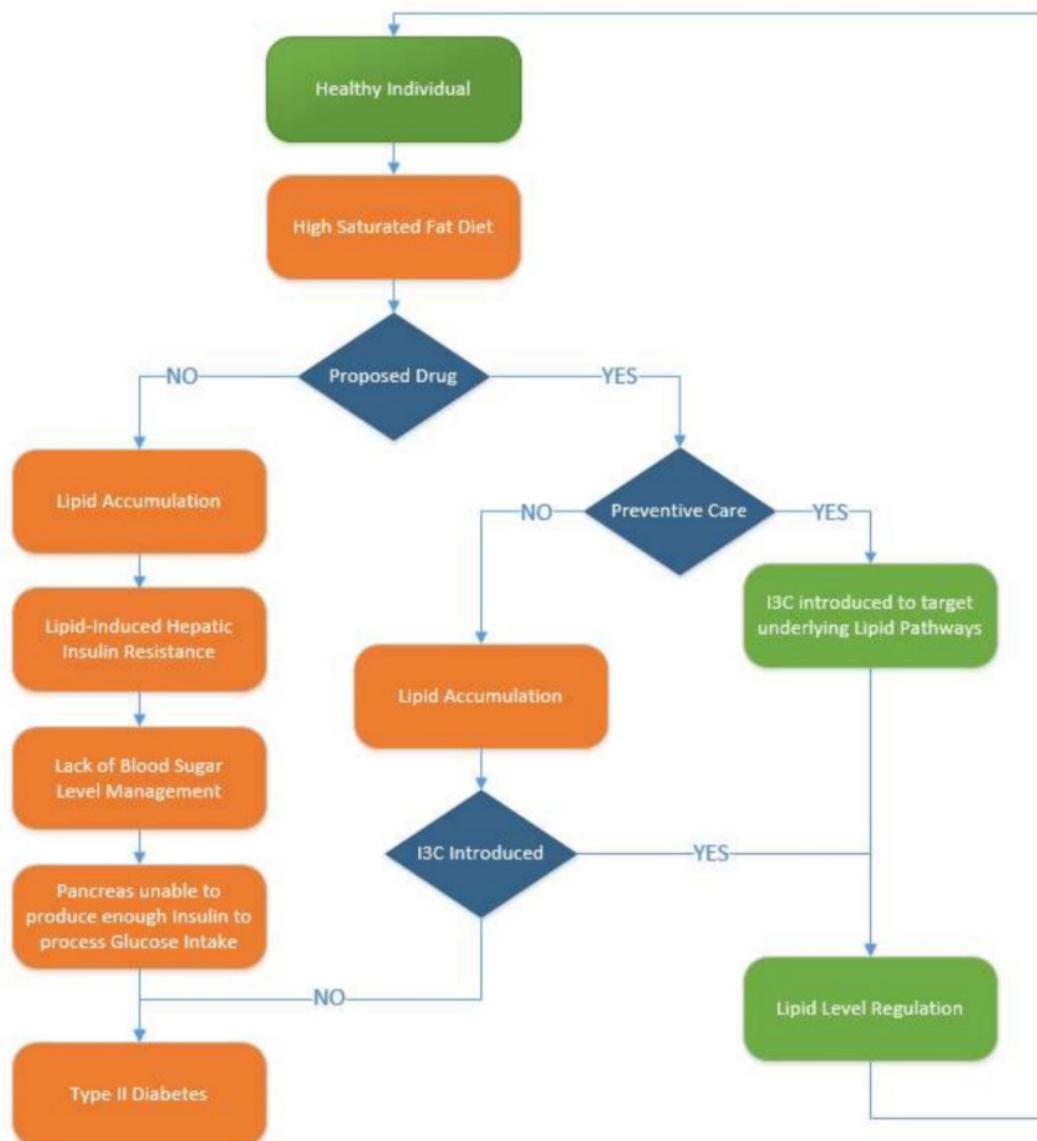
Figure 3. A schematic representation of the effect of the proposed treatment according to the results observed on C. elegans in this study. Flow chart presenting development of T2D diabetes naturally on left hand side in comparison to the potential effects of the proposed drug with the results observed in this study shown on the right side. Preventative care treatment route shown with I3C treatment outlined graphically.

brightness, and the integrated density of the image, which takes into consideration the sum of the brightness of all the pixels inside the selected worm outline. As a comparison, a small circle outlining a portion of the background was drawn to determine the mean brightness of the background.

The CTAF values were then calculated for each worm, and the average CTAF values for each experimental group were determined by adding all 10 calculated CTAF values then dividing by 10 (the number of data points per experimental group of 1 trial), so that there were 4 average CTAF values per trial to be compared amongst each other.

Analyzing for Statistical Significance

Average CTAF values across each experimental group were evaluated for statistical significance using an analysis of variance test (ANOVA) and an honestly significant difference test (Tukey HSD). The statistical comparisons were made using a One-way ANOVA and post-hoc Tukey HSD Test online - Calculator by Navendu Vasavada (26). The outputted *p*-value is representative of the probability that an observed difference in experimental groups is due to chance; a value of less than 0.05 is considered statistically significant.

ACKNOWLEDGEMENTS

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