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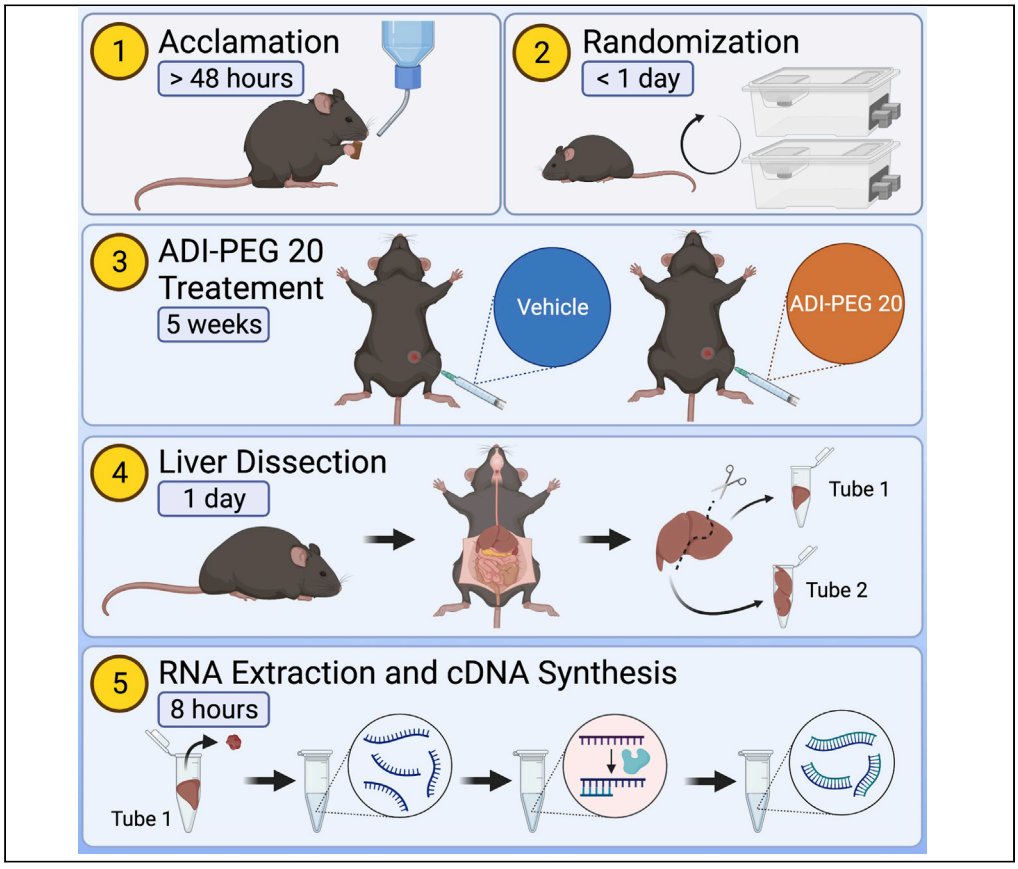
# A protocol to induce systemic autophagy and increase energy metabolism in mice using PEGylated arginine deiminase




Obesity is a prevalent metabolic disorder worldwide. Here, we describe a comprehensive protocol using pegylated arginine deiminase (ADI-EPG 20) to apply the concept that arginine depletion induces systemic autophagy to drive whole-body energy metabolism and weight loss in mice. We detail the steps for cohort setup, mouse husbandry, and treatment and provide expected results under these conditions.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Highlights

Protocol to study the role of systemic arginine depletion in obesity via ADI-PEG 20

Description of ADI-PEG 20 treatment and mouse husbandry to ensure reproducible results

A reference protocol for investigating other physiological effects of ADI-PEG 20

Method for examining other diseases stemming from autophagy insufficiency

Zhang & DeBosch, STAR Protocols 3, 101489
September 16, 2022 © 2022 The Author(s).
https://doi.org/10.1016/j.xpro.2022.101489

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# A protocol to induce systemic autophagy and increase energy metabolism in mice using PEGylated arginine deiminase

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https://doi.org/10.1016/j.xpro.2022.101489

## SUMMARY

**Obesity is a prevalent metabolic disorder worldwide. Here, we describe a comprehensive protocol using pegylated arginine deiminase (ADI-EPG 20) to apply the concept that arginine depletion induces systemic autophagy to drive whole-body energy metabolism and weight loss in mice. We detail the steps for cohort setup, mouse husbandry, and treatment and provide expected results under these conditions.**

**For complete details on the use and execution of this protocol, please refer to Zhang et al. (2022a, 2022b).**

## BEFORE YOU BEGIN

The protocol below describes the specific steps for setting up the mouse cohorts and the induction of obesity prior to ADI-PEG 20 treatment in genetically obese *db/db* mice. ADI-PEG 20 is an enzyme that rapidly depletes extracellular arginine. It has been studied in numerous types of arginine auxotrophic cancer, including hepatocellular carcinoma (Abou-Alfa et al., 2018; Patil et al., 2016). In addition, we recently demonstrated that ADI-PEG 20 also improves insulin and glucose tolerance in genetically obese mice, in part, by driving systemic autophagic flux (Zhang et al., 2022a, 2022b). Therefore, we describe in this protocol the use of ADI-PEG 20 to treat metabolic disease in diet-induced and genetically obese mouse models, and subsequent quantification of key outcome measures (Zhang et al., 2022a).

### Institutional permissions

All animal procedures described in this protocol were approved by the Institutional Animal Care and Use Committee (IACUC) at the Washington University School of Medicine under protocol ID 20-0330. All experiments described in this protocol were conducted in compliance with the ethical guidelines and regulatory policies of the NIH. Mice were obtained from Jackson Laboratory and maintained with standard mouse chow and water ad libitum.

### Setup for genetically obese mice

Timing: 3 days–1 week

1. Obtain approval from the Institutional Animal Care and Use Committee prior to starting any animal work.


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2. 5 wk-old genetically obese *db/db* mice (*Lepr*$^{db}$, genotype: *a/a* + *Lepr*$^{db}$/+ *Lepr*$^{db}$, Cat# 000697) were obtained from the Jackson Laboratory.
3. *db/db* mice arrive at age 5 weeks, with a range of ± 3 days per supplier specification.
4. House *db/db* mice in cages of no more than 4 mice per cage in a temperature-controlled room with a 12-h alternating light/dark cycle in a specific pathogen-free barrier facility prior to and throughout experimentation.
   a. Temperature: 22°C ± 1°C.
   b. Light cycle: 6 AM–6 PM; Dark cycle: 6 PM–6 AM.
5. Feed mice with a regular chow diet with sterile, autoclaved drinking water.
   a. Specific care is taken to assure no leakage from water bottle seals, as cage flooding confounds subsequent metabolic and morphologic data.
6. If mice are obtained through a vendor and delivered to the facility where the experiment will be carried out, a minimum of 48 h post-delivery should be given to the animals for acclimation prior to further manipulation.

   ***Note:*** Both female and male mice may be used. In general, male *db/db* mice gain weight more rapidly and present metabolic phenotypes that are more severe than female mice.

7. Randomly assign mice to vehicle control (saline) or ADI-PEG 20 treatment group.
8. House four mice per cage.

   ***Note:*** Researchers can adjust the number of mice per cage based on preference. *db/db* mice are polyphagic and polyuric. Thus, the cage can get crowded and extremely messy even with daily cage changes (Figure 4). We prefer a maximum of four *db/db* mice per cage to limit the crowding and to maximize cage cleanliness.

9. Generate unique identification of a mouse by either ear tag or ear punch.
10. Assign identification numbers to every animal enlisted in the experimental cohort and obtain the initial body weight and body composition of every animal.
11. Perform a two-tailed Student's *t*-test on the body weights between control and treatment groups to make sure no statistical difference in mean body mass exists between the groups prior to experimentation (Figure 1A).
12. If *p*-value < 0.05, carefully check the mean and standard deviation of the body weight. We recommend re-randomization of mice between treatment/cage to ensure even balanced body mass distribution across groups at final enrollment (Figure 1B).

    ***Note:*** We have empirically determined that this process minimizes variability in outcome measures due to basal differences.

    ***Note:*** Experimentalists may choose to stratify the randomization based on body weight, body composition, or both. Generally, re-randomization of *db/db* mice based solely on body weight will result in an evenly balanced body composition of fat and lean mass (Figures 1C and 1D).

13. Finalize the control and treatment groups based on the adjusted assignment. The higher the *p*-value, the better.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, peptides, and recombinant proteins | | |
| ADI-PEG 20 | Polaris Pharmaceuticals Inc. | Kit# 36386 |
| 0.9% Sodium Chloride Injection, USP | Hospira | NDC# 0409-4888-02 |

(*Continued on next page*)

| *Continued* | | |
|---|---|---|
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| TRIzol | Invitrogen | Cat# 15596018 |
| Chloroform | Sigma-Aldrich | SKU 319988-500ML |
| 2-Propanol | Sigma-Aldrich | SKU 190764-500ML |
| Methanol | Sigma-Aldrich | SKU 179337-4L-PB |
| Ethanol | Decon Laboratories, Inc. | Cat# 2701 |
| Nuclease-Free Water | Invitrogen | Cat# AM9937 |
| Critical commercial assays | | |
| QuantiTect Reverse Transcription Kit | QIAGEN | Cat# 205314 |
| Experimental models: Organisms/strains | | |
| Mouse: *db/db*; male animals; 5–10 weeks old (juvenile to adulthood) | The Jackson Laboratory | Cat# 000697; RRID: IMSR_JAX:000697 |
| Software and algorithms | | |
| Microsoft Excel | Microsoft | https://www.microsoft.com/en-ww/microsoft-365/excel |
| GraphPad Prism 7 | GraphPad Software Inc. | http://www.graphpad.com |
| Other | | |
| PicoLab Rodent Diet, Irradiated | LabDiet | Cat# 52WU |
| Nestlets nesting material-mice | Ancare Corporation | Cat# NC9365966 |
| Syringe with needles | McKesson | Cat# 942674; Mfr# 102-SN30C3516P |
| Weighing dish | Fisher Scientific | Cat# 02-202-100 |
| Analytical balance | Fisher Scientific | N/A |
| Disposable pestle grinders, polypropylene, 1.5 mL | Kimble Chase | Cat# 749521-1500 |
| Nanodrop One spectrophotometer | Thermo Scientific | Cat# ND-ONE-W |
| T100 Thermal Cycler | Bio-Rad | Cat# 1861096 |
| Surgical scissors | Fine Science Tools | Cat# 14058-11 |
| Dumostar tweezers, #7, high precision grade | Dumont | Cat# 10579 |
| EchoMRI 3-in-1 body composition analyzer | Echo Medical Systems | N/A |

## MATERIALS AND EQUIPMENT

Additional thermal cycler setup for genomic DNA (gDNA) elimination and reverse transcription is provided below.

| **Thermal cycler setup for genomic DNA (gDNA) wipeout reaction** | | |
|---|---|---|
| Steps | Temperature | Time |
| 1 | 42°C | 4 min |
| 2 | 4°C | ∞ |

| **Thermal cycler setup for reverse-transcription reaction** | | |
|---|---|---|
| Steps | Temperature | Time |
| 1 | 42°C | 20 min |
| 2 | 95°C | 3 min |
| 3 | 4°C | ∞ |

*Note:* We recommend taking the samples out and immediately place on ice after each reaction.

*Note:* The incubation time on some steps is slightly modified from the manufacturer's protocol.

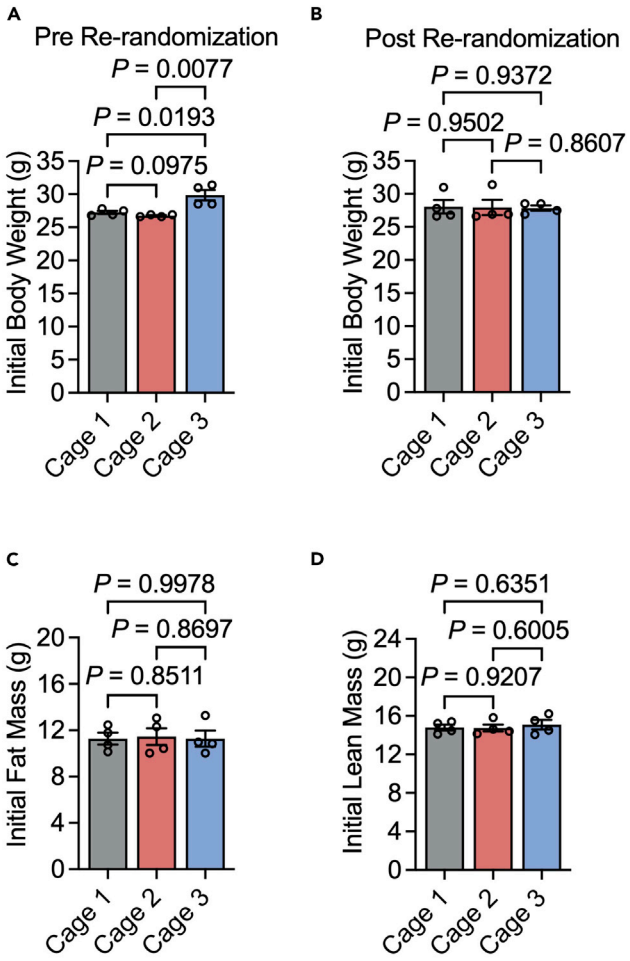


**Figure 1. Example body weight distribution post randomization during experimental cohort setup**

(A) Body weights of 12 *db/db* mice after randomly assigning to three cages (n=4 mice per cage).

(B) Body weights of the same mice after body weight-based re-randomization into three cages.

(C) Fat mass measured by EchoMRI.

(D) Lean mass measured by EchoMRI.

Data represented in mean ± SEM (n=4 mice per cage). Each data point represents an individual animal. Exact *p*-values are shown. Statistical significance was determined using unpaired two-tailed Student's *t*-test in (A), (B), (C), and (D).

*Note:* Store cDNA samples at −20°C for long-term storage. Avoid repeated freeze-thaw cycles.

## STEP-BY-STEP METHOD DETAILS

To induce arginine depletion, we delivered ADI-PEG 20 weekly via intraperitoneal injection (IP) into genetically obese *db/db* mice, and age-matched littermate *db/db* mice are used as controls. The following procedure is written for a 5-week treatment regimen. Although we have not yet dosed over a longer period of time, prior mouse and human studies with treatment duration up to 13 weeks have been reported without serious drug-related adverse events (Hajji et al., 2022; Tsai et al., 2017).

### ADI-PEG 20 treatment

Timing: 5 weeks

After the animals are acclimated and experimental groupings are finalized, initiate ADI-PEG 20 treatment.

1. Take mice cages one at a time to a dedicated biosafety cabinet for animal procedures.
2. Remove the cage lid, food, and water bottle from the cage.
3. Measure the body weight of every mouse in the cage and record the weights.
4. Prior to ADI-PEG 20 injection, obtain a single-use syringe with needle and draw the necessary volume for injection.
   a. Deliver 5 IU/animal intraperitoneally (IP). In our case, we injected 50 μL per animal at protocol doses.
   b. For control animals, IP inject equal volumes (50 μL) of 0.9% saline per animal.

   *Note:* Stock ADI-PEG 20 solution is clear and colorless. ADI-PEG 20 is prepared and shipped in small glass vials. Each vial contains 3.5 mL solution with a protein concentration of ~11.0 mg/mL. ADI-PEG 20 should be stored at −80°C, and aliquoted to avoid repeated freeze-thaw cycles. To prepare working stocks of ADI-PEG 20 for treatment, we thawed 1 vial of stock ADI-PEG 20 and aliquoted the undiluted contents into separate tubes. The volume of the aliquot is calculated to match the number of mice we need to inject, such that we only use 1 aliquot per round of treatments.

   *Optional:* ADI-PEG 20 can be diluted and injected on the day of treatment. The stock material is slightly more viscous than water, but is highly miscible with other diluents, such as 0.9% saline solution.

   ⚠ CRITICAL: Experimentalists should optimize delivery volume based on the lot/stock concentration of ADI-PEG 20 available. The timing of the weekly ADI-PEG 20 injection must be kept consistent every week. For example, our ADI-PEG 20 treatments in *db/db* mice occurred every Wednesday at 8:00 AM ± 30 min.

5. After injection, slowly and carefully place the animal into a fresh cage with new bedding and nestlets (two squares per cage).
6. Once each animal has been treated, weigh the remaining water and rodent chow. Freshly replenish food and water into the cage, and secure the lid.
   a. To measure food and water consumption, weigh the residual chow and measure the amount of water left 24 h (or a time interval of choice) later using a graduated cylinder.
   b. Subtract the leftover food weight and water volume by the initial to get the total consumption per cage.
   c. Individual mouse consumption can be estimated by dividing the total cage consumption by the number of animals per cage.
7. Return the cage to its original precise position within the housing suite, such that the visual environment of the mouse remains consistent throughout the trial. Move on to the next cage and repeat steps 3–6.
8. Monitor body weight, food consumption, water consumption, and record daily.

   *Note:* Daily monitoring of body weight, food consumption, and water consumption are not necessarily required. Adjust the monitoring frequency based on research interest and goal. However, we recommend daily monitoring as the animals become more polydipsic and polyuric with age and advancing obesity.

   *Note:* Depending on the experimental design, experimentalists can eliminate food intake as a variable by pair-feeding, or through food access restriction.

⚠ CRITICAL: Experimentalists should maintain consistent time-of-day for measurements, regardless of the monitoring frequency adopted for their experiment. For example, body weight measurements in ADI-PEG 20-treated *db/db* mice occurred daily at 8:00 AM ± 30 min in 24-h intervals. This is because mouse body weight fluctuates throughout the day. This body weight fluctuation is often further pronounced in obese mice when compared with lean mice.

9. Body composition can be measured depending on the availability of an echoMRI.

### Liver dissection

Timing: 1 day

The following protocol describes in detail the steps for mouse euthanasia, liver dissection, and tissue handling. Liver dissection for the entire cohort can generally be completed in one day. Depending on the size of the cohort, animal sacrifice can occur in parallel with the help of lab mates.

10. Sacrifice mouse 48 h after the last ADI-PEG 20 injection.

⚠ CRITICAL: Tissue collection for each mouse should be completed 48 h ± 30 min after the last ADI-PEG 20 injection.

11. Euthanize mouse by cervical dislocation.

*Note:* Other approved methods of euthanasia with or without a secondary physical method of euthanasia to ensure death are also acceptable, as long as the person/people performing the procedures are fully trained.

12. Spray the mouse fur with 70% ethanol to prevent fur contamination into dissected tissues.
13. Place the mouse in a supine position on the dissection table.
14. Locate the abdomen and make a small longitudinal incision along the midline and expose the peritoneal sheath.
15. Carefully make another small incision on the parietal peritoneum without damaging any internal organs.
16. From the small incision, make an inverted Y- or inverted T-shaped incision connecting to the xiphoid process of thorax to expose the abdominal cavity and viscera (Figures 2A and 2C).

*Note:* Experimentalists can also make a horizontal incision along the inferior aspect of the 12$^{th}$ rib to expose the entire liver.

17. Identify the left lateral lobe of liver (Figure 2). Gently lift the left lateral lobe using a pair of surgical forceps and excise at the point of attachment (Figure 2D, marked by white bold line).

*Optional:* The gallbladder sits just underneath the right medial and the left medial lobe of the liver (Figures 2B and 2D). Avoid gallbladder rupture during liver dissection. To achieve this, carefully remove the gallbladder first by using forceps to grip the common bile duct. Cut and lift away the entire structure after incising the common bile duct, with the gallbladder fully intact.

18. Coarsely cut the left lateral lobe into small pieces and place all liver pieces in a labeled 1.7 mL tube.
19. Immediately snap-freeze in liquid nitrogen.

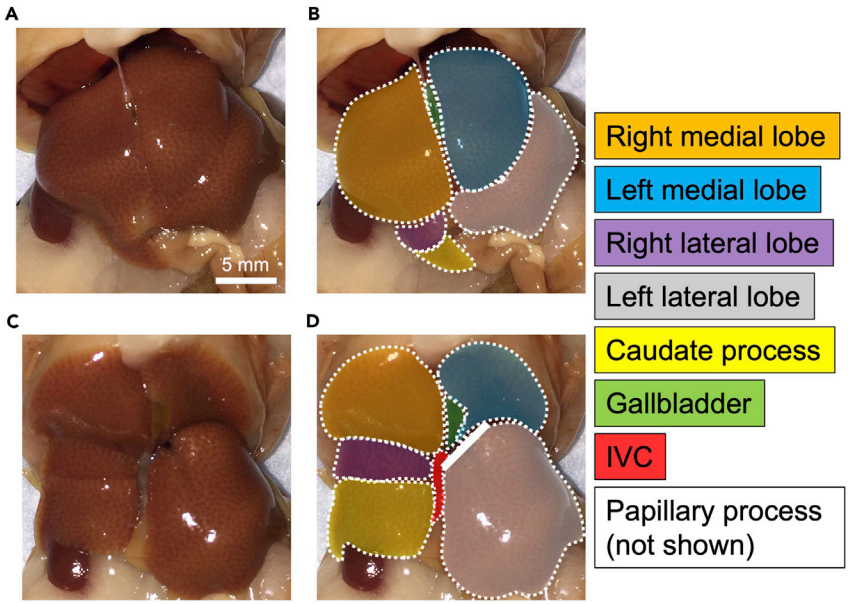


**Figure 2. Illustration of murine liver lobes to aid lobe identification during liver dissection**

(A) Ventral view of a male *db/db* mouse liver. Scale bar, 5 mm.

(B) Figure (A) with corresponding liver lobes outlined by dot line and color coded.

(C) Ventral view of the same male *db/db* mouse liver with the medial lobe folded cranially to reveal the liver lobes underneath.

(D) Figure (C) with corresponding liver lobes outlined by dot line and color coded. The white bold line marks the excision line to remove the left lateral lobe (gray).

Right medial lobe (orange), left medial lobe (blue), right lateral lobe (purple), left lateral lobe (gray), caudate process (yellow), gallbladder (green), and inferior vena cava (IVC, red). The papillary process is located underneath the left lateral lobe (gray) and is not depicted in this figure.

20. Remove the remaining liver lobes. Quickly place the liver tissue in a separately labeled 1.7 mL tube and immediately snap-freeze in liquid nitrogen.

*Optional:* If liver weight is desired, excise out all lobes of liver first. Zero the weight on an analytical balance with a small 1″ disposable weighing dish.

*Optional:* Other tissues of interest can also be harvested. If additional tissues are desired, experimentalist should incorporate sufficient dissection time for the additional tissues.

⚠ CRITICAL: When collecting multiple tissues, tissues that are more metabolically active, such as liver and heart, should be prioritized for rapid collection. Subcutaneous adipose tissue and visceral adipose tissue that are less metabolically active can be collected last.

21. Store all tissue samples at −80°C.

**Pause point:** Liver and other tissues can be left at −80°C for long-term storage.

## RNA extraction and cDNA synthesis

**Timing: 8 h**

The following protocol describes in detail the steps for RNA extraction from murine liver tissue. RNA extraction and cDNA synthesis can be completed in one day or split into multiple days. Briefly, liver

tissue is isolated using TRIzol™ Reagent (Invitrogen). Complementary DNA (cDNA) is synthesized using QuantiTect® Reverse Transcription Kit (Qiagen).

22. 25–35 mg of liver tissue collected from step 19 of this protocol is used for RNA extraction.
23. Use 750 μL of TRIzol Reagent per sample for RNA extraction in a prelabeled 1.7 mL tube.
24. Homogenize the liver tissue in TRIzol Reagent with a 1.5 mL KONTES® PELLET PESTLE® Grinder (Kimble Chase).
25. Perform RNA extraction using TRIzol Reagent by following the manufacturer's instructions (Detailed protocol can be found in the following link: TRIzol™ Reagent User Guide).
26. Use a spectrophotometer to measure the concentration of extracted RNA.
27. Synthesize cDNA from extracted RNA using QuantiTect Reverse Transcription Kit by following the manufacturer's instructions (Detailed protocol can be found in the following link: QuantiTect® Reverse Transcription Kit Quick-Start Protocol).
28. Reverse transcription reaction components and reaction setup is described in the materials and equipment section of this protocol.

⚠ CRITICAL: Use sterile tubes that are certified RNase- and DNase-free, and sterile, low-binding, filtered tips that are certified RNase- and DNase-free for all steps described.

## EXPECTED OUTCOMES

### Expected outcomes of ADI-PEG 20 treatment on weight gain

Successful completion of this protocol will result in robust attenuation of weight gain in *db/db* mice. The effect of ADI-PEG 20 treatment on body weight can be seen within days after a single injection. Body weight measurements may have an upward trend up to 48 h post ADI-PEG 20-treatment, but weight gain will eventually plateau between 48 to 96 h post-treatment (Figure 3A).

### Expected outcomes of ADI-PEG 20 treatment on body composition

Conversely, at the end of the 5-week treatment, ADI-PEG 20 treated animals exhibit significantly reduced fat:total mass ratio and concomitantly increased lean:total body mass percentage (Figure 3B). The drastic change in percent body composition results from significant loss of absolute fat mass without significant changes in lean mass (Figure 3C).

### Expected outcomes of ADI-PEG 20 treatment on behaviors

*db/db* mice are polyphagic, polydypsic, and polyuric. Increases in appetite, thirst, and urination, respectively, cause weight gain to occur at a rapid rate (~1 g/day). Of the three conditions, polyuria is often an earlier symptom for these diabetic mice, providing an easily identifiable (direct) visual clue of deteriorating health. ADI-PEG 20-treatment attenuates the onset of polyuria in *db/db* mice 2 weeks into the regimen (Figure 4A). At 5 weeks, polyuric progression was attenuated in ADI-PEG 20-treated mice compared to controls (Figure 4B).

Healthy rodents exhibit repetitive behaviors such as grooming and roaming. Changes in repetitive behaviors often indicate illness and disease (Richardson, 2015). Nestlet shredding is a simple assay used in behavioral analysis (Dorninger et al., 2020). Obese control *db/db* mice progressively lose interest in nestlet shredding, whereas the shredding behavior sustained in ADI-PEG 20 treated mice (Figure 4B).

### Expected outcomes of RNA extraction and RNA quality

Extraction of total RNA using TRIzol Reagent usually yields A260/280 values between 1.950–2.100 and A260/A230 values between 1.750–2.200.

For more information on the expected outcomes of other metabolic parameters not described in this protocol, please refer to Zhang et al. (2022a).

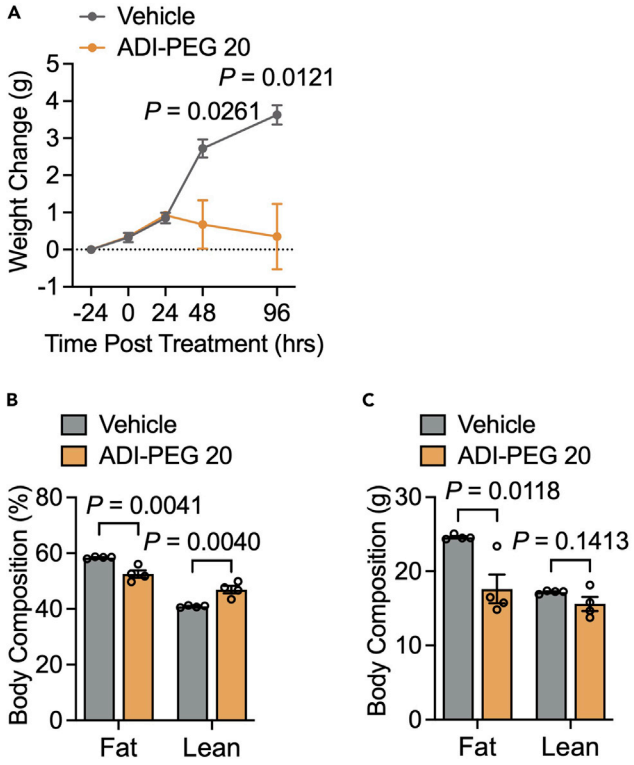


**Figure 3. The effect of ADI-PEG 20 treatment on body weight and body composition**

(A) Change in total body weight 24 h before to 96 h after a single ADI-PEG 20 injection of 5 IU/mouse (n=4 mice per group).

(B) Percent body composition of fat and lean mass.

(C) Absolute body composition of fat and lean mass.

Data represented in mean ± SEM (n=4 mice per group). Each data point represents an individual animal. Exact *p*-values are shown. Statistical significance was determined using unpaired two-tailed Student's *t*-test in (A), (B), and (C). Data presented in (B) is reprinted with permission from Zhang et al. (2022a, 2022b).

## QUANTIFICATION AND STATISTICAL ANALYSIS

Mice should not be excluded unless unexpected health-related issues arise that will affect the animal metabolically. For example, ulcerative dermatitis (UD) that is refractory to antibiotic topical ointment can cause rapid weight reduction.

For direct comparison between groups, the standard two-tailed Student's *t*-test is sufficient to test for statistical significance. Raw data generated during experimentation can be imported and stored in Excel for the downstream analysis process. Data can also be easily imported into other data analysis and visualization software, such as GraphPad Prism.

Unless otherwise specified, statistical analysis throughout this protocol was done by two-tailed Student's *t*-test with the exact *p*-value listed. All error bars represent SEM unless otherwise stated.

## LIMITATIONS

In this protocol, we describe how to set up mouse cohort and how to perform ADI-PEG 20 treatments with precautions towards introducing non-metabolic variations. Currently, the protocol describes a five-week ADI-PEG 20 treatment regimen with a weekly IP injection of 5 IU/mouse. We empirically tested the tolerance and dosage response of ADI-PEG 20 by administering 5 IU/animal once vs. twice weekly. Weight gain is fully attenuated in the high-dose group (5 IU × 2/animal/week)

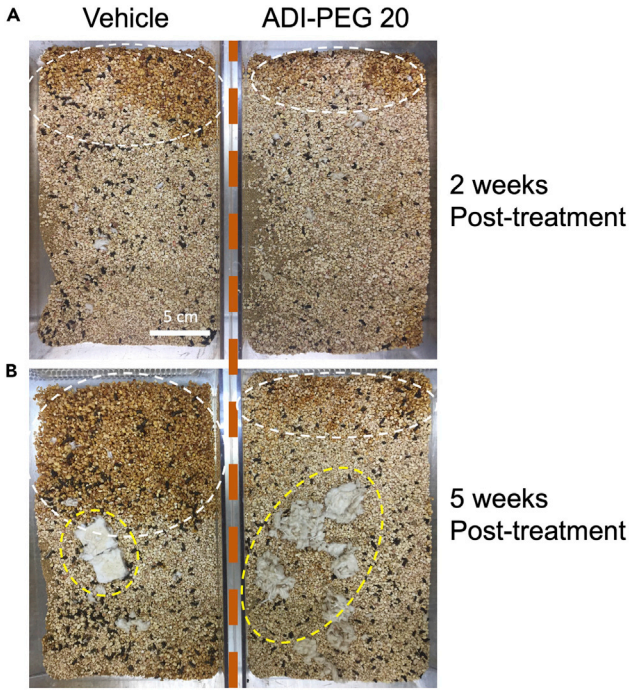


**Figure 4. The effect of ADI-PEG 20 treatment on polyuria and nestlets shredding behavior**

(A) Cage appearance 24 h post new cage change after 2 weeks of ADI-PEG 20 treatment in *db/db* mice. Scale bar, 5 cm.

(B) Cage appearance 24 h post new cage change after 5 weeks of ADI-PEG 20 treatment in *db/db* mice.

Each cage was used to house 4 *db/db* mice. Cobb bedding soaked in urine is circled and outlined with white dashes in (A) and (B). Nestlets are circled with yellow dashes in (B).

(Figure 5A). Upon examination of body composition at 2 weeks post-treatment, the two-dose per week group exhibited a significant reduction in both fat and lean mass, whereas the one-dose per week group exhibited only reduced fat mass (Figures 5B and 5C) in the absence of lean mass changes. Therefore, we elected to use 5 IU/mouse/week for our metabolic experiments. This corroborates prior experiments using 5 IU per mouse per week ADI-PEG 20 in the cancer treatment protocols (Ji et al., 2020; Miraki-Moud et al., 2015; Sahu et al., 2017).

This protocol provides a platform/model to study the underlying mechanisms of energy and arginine homeostasis and can be used to further examine the efficacy of arginine targeting in other diseases that stem from autophagic insufficiency. However, the current protocol utilized only a single dosage of ADI-PEG 20 and did not optimize ADI-PEG 20 dosage based on other metabolic parameters such as body weight. Previously, ADI-PEG 20 treatment durations range from 1 to 4 weeks in studies of its antitumor functions. Our study on the metabolic treatment duration of 5 weeks was optimized for metabolic studies. Although ADI-PEG 20 in a clinical trial may continue for up to 103 weeks, the weekly dosage used in the human trial is based on the area (36 mg/m$^2$) (NCT03449901). Therefore, additional *in vivo* experiments in mice should be performed to determine the optimal dosage and duration given specific experimental goals.

## TROUBLESHOOTING

### Problem 1

ADI-PEG 20 solution is too viscous. Cannot deliver the correct amount of ADI-PEG 20 due to bubble formation in the syringe. (step 1 to "step-by-step method details").

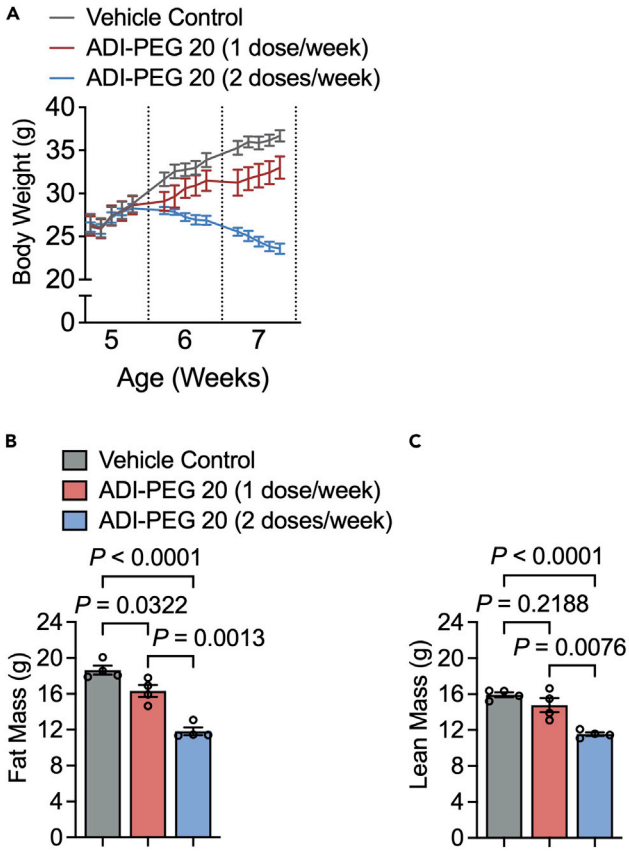


**Figure 5. ADI-PEG 20 dose response in *db/db* mice post 2-week treatment**

(A) Body weights of *db/db* mice treated with and without ADI-PEG 20 for 2 weeks (n=4 mice per group).

(B) Total fat mass of *db/db* mice treated with and without ADI-PEG 20 for 2 weeks.(C) Total lean mass of *db/db* mice treated with and without ADI-PEG 20 for 2 weeks.

Data represented in mean ± SEM (n=4 mice per group). Each data point represents an individual animal. Exact *p*-values are shown. Statistical significance was determined using unpaired two-tailed Student's *t*-test in (A), (B), and (C).

## Potential solution

Stock ADI-PEG 20 solution has a higher viscosity than water. Using a syringe with a larger gauge needle can help avoid excess trapping of air bubbles in the syringe. An alternative method is to dilute the stock ADI-PEG 20 solution with 0.9% saline solution before injection on the day of treatment. ADI-PEG 20 is easily miscible with 0.9% saline solution. If a larger volume of diluted ADI-PEG 20 is used to deliver 5 IU of ADI-PEG 20, remember to also increase the volume of saline for the control group.

## Problem 2

The animal does not respond to ADI-PEG 20 treatment. Attenuated weight gain is not observed 48 h post-injection (step 4 in "step-by-step method details").

## Potential solution

Check proper technique for IP injection. Inconsistent drug delivery site or ectopic delivery, for example to the liver, or urinary bladder, may underlie inconsistent dosing.

## Problem 3

Animals respond to ADI-PEG 20 treatments differently and seem to be in a weight-dependent way. A reduced effect is observed in heavier animals (step 8 in "step-by-step method details").

### Potential solution

ADI-PEG 20 dosing may need to be optimized based on body weight. The dosages reported here are specific to *db/db* animal weights ranging ~25–35 g.

### Problem 4

Some metabolic effects of ADI-PEG 20 are not observed (step 10 in "step-by-step method details").

### Potential solution

Adjust the timing of phenotypic experiments or tissue dissection. Although ADI-PEG 20 has a half-life of 6–8 days (Thomas et al., 2002), we observe attenuation in weight gain 24–96 h post-injection (Figure 3A). Thus, we recommend performing metabolism-related phenotypic experiments within the window between 48 to 96 h post-treatment if able.

### Problem 5

Inconsistent animal behavior and metabolic phenotype (related to "expected outcomes").

### Potential solution

Make sure animals are housed in rooms with minimum noise or visual disturbance. Ensure that polyuric animal cages are changed at least daily. This is necessary to maintain a clean living environment and reduce animal stress, which can affect some metabolic parameters. Ensure that no water bottle leakage or flooding has occurred. Ensure that your handling of each animal is slow, calm, and equal across every animal as a stress minimization measure.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Brian J. DeBosch (deboschb@wustl.edu).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate any new dataset or code for analysis.

## ACKNOWLEDGMENTS

This work was supported by grants from the NIDDK (1R01DK126622-01A1, B.J.D.), NHLBI (1R01HL147968-01A1, B.J.D.), AASLD (Pilot Research Award, B.J.D.), NCCIH (1R21AT010520-01, B.J.D.), NIH/National Center for Advancing Translational Sciences (NCATS, #UL1TR002345, B.J.D.), NIDDK (R56DK115764, B.J.D.), AGA-Gilead Sciences Research Scholar Award in Liver Disease (B.J.D.), the AGA-Allergan Foundation Pilot Research Award in Non-Alcoholic Fatty Liver Disease (B.J.D.), the Washington University Digestive Disease Research Core Center (P30DK052574), Washington University Diabetes Research Center (P30DK020579), the Nutrition & Obesity Research Center (P30DK056341), the Association for Aging Research Junior Faculty Award (B.J.D.), the Robert Wood Johnson Foundation (B.J.D.), the Washington University Center for Autophagy Therapeutics Research (B.J.D.), and the Longer Life Foundation (B.J.D.). Y.Z. is a pre-doctoral fellow supported by the Washington University School of Medicine Pediatric Gastroenterology Research Training Grant (T32DK077653), and the Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship (F31DK131875). ADI-PEG 20 was a kind gift from Dr. John S. Bomalaski (Polaris Pharmaceuticals, Inc., San Diego, CA, USA).

## AUTHOR CONTRIBUTIONS

Conceptualization, Y.Z. and B.J.D.; Methodology, Y.Z.; Investigation, Y.Z.; Data Curation, Y.Z.; Formal Analysis, Y.Z.; Visualization, Y.Z.; Pictures, Y.Z.; G.A. Graphics, Y.Z.; Writing – Original Draft,

Y.Z.; Writing – Review and Editing, Y.Z. and B.J.D.; Supervision, B.J.D.; Project Administration, Y.Z. and B.J.D.; and Funding Acquisition, Y.Z. and B.J.D.

## DECLARATION OF INTERESTS

Part of this study was funded by a sponsored research agreement awarded by Polaris Pharmaceuticals (to B.J.D.). B.J.D. is the lead inventor on US Patent Application #17/050,318. Relevant US Patent Publication #US2021/0077598, toward which the presented data are material.

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