Supplementary Materials for

Controlled-Release Mitochondrial Protonophore Reverses Diabetes and Steatohepatitis in Rats

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Materials and Methods

Study design

The objective of this research was to test the hypothesis that administering DNP in a controlled-release manner would improve its safety versus unaltered DNP without compromising its efficacy to treat NAFLD, insulin resistance, type 2 diabetes, and liver fibrosis. We tested this hypothesis in male rats and mice that were randomized to treatment groups after ensuring that the randomization would allow body weight to be matched between the groups prior to the start of the experiment. The researchers were not blinded in all experiments, but the histological analysis and measurement of plasma and tissue DNP concentrations were performed by blinded investigators.

Sample size was chosen before the studies were undertaken to assess prospectively determined endpoints based on a power calculation. We expected differences of at least 20% between groups, with standard errors 15% of the mean. The required sample size was calculated to be 5; to have sufficient leeway to detect differences, we used sample sizes of 6-8 for most experiments. In rare cases a rat was excluded due to technical issues: for instance, a rat whose arterial and venous catheters did not remain patent after surgery could not be used for glucose tolerance test or hyperinsulinemic-euglycemic clamp studies. No data or animals were excluded due to being outliers, and no experiments were terminated prematurely. Although certain experiments dictated that a measurement be taken multiple times through the course of one experiment (e.g. body temperature time courses, Fig. S2), each discrete experiment was performed once on each animal and no other sampling replicates were performed.

Formulation of CRMP

DNP was selected as the protonophore in these experiments because it is the best studied mitochondrial uncoupler (for instance, a PubMed search for "2,4-dinitrophenol" yields 3620 results, as compared to 2155 for "carbonyl cyanide m-chlorophenyl hydrazine (CCCP) and 92 for "carbonylcyanide p trifluoromethoxyphenylhydrazone (FCCP)"). The simple structure and relatively low molecular weight of DNP as compared to other mitochondrial uncouplers also recommends it as an oral agent, reducing dosing limitations due to the weight of the drug.

In order to reduce toxicity while maximizing efficacy, we contracted with Emerson Resources to develop a 2,4-DNP extruded or drug layered bead which could further be coated with a sustained release coating in a dosage size suitable to deliver 1.0 mg/kg of 2,4-DNP over a 12-24 hour period. The final goals were to:

- Develop an extruded or drug layered 2,4-DNP bead suitable for coating
- Evaluate various polymers or polymer combination which would deliver a 12-24 hr release of 2,4-DNP
- Ensure the dosage is suitably sized for delivering 1.0 mg/kg to rodents

The polymers selected for the sustained release coating work independent of pH, as they are a combination of soluble and insoluble polymers, allowing release through pores created by the soluble polymer; thus the drug may be absorbed in the acidic stomach and/or neutral intestine. Initially, Emerson focused on developing a substrate (2,4-DNP extruded or drug layered bead) on which to apply a sustained release coating. A multiparticulate system was selected because the more particulates that are coated, the less likely variances in dissolution occur (as a coating failure in a tablet could result in

complete release of the dose whereas a coating failure in one of many particulates would result in a minimally altered dissolution profile). However, initial assessment of drug solubility in water and various solvents (alcohols) proved poor and the feasibility of drug layering onto an inert substrate (sugar or MCC spheres) was eliminated. Because of this, extrusion and spheronization was selected as the process for creating a 2,4-DNP substrate (bead).

In order to achieve controlled release, two polymer systems were selected for evaluation: a Eudragit RS/RL system and a hydroxypropylcellulose (HPC)/ethylcellulose (EC) system. Initial formulation work focused on evaluating the HPC/EC and Eudragit systems at various coating levels. With immediate success at achieving a desirable release profile with the HPC/EC system and challenges observed (blocking) with the Eudratig system, the HPC/EC system was selected as the preferred coating system for continued work.

The following table shows the raw materials used for preparation of CRMP spheres:

Generic Name	**Trade Name**	**Supplier**
Mannitol	Mannitol 100 SD	Roquette
Microcrystalline cellulose (MCC)	MCC PH-101	FMC Biopolymer
Hydroxypropylmethyl cellulose (HPMC)	Pharmacoat 606	Shin-Etsu
Ethylcellulose (EC)	ECN10	Dow
Hydroxypropylcellulose (HPC)	Klucel EF	Ashland
GMS Emulsion	Plasacryl T20	Emerson
Polysorbate 80	---	Evonik
Talc	---	Brenntag
Dibutylsulfate	---	Vertellus
Distilled water	---	Emerson
Ethanol	Ethanol 200 proof	Spectrum

Uncoated 2,4-DNP Extruded Sphere Development

Emerson selected a desired dose weight based on three factors: developing a dosage weight that would feasibly deliver the requested dose (1.0-1.5 mg/kg) to rodents, to avoid uniformity issues, and to avoid issues with the sustained release coating. Based on the selected dose weight and API concentration, a formulation using common excipients for extruded beads was developed. The initial bead formulation (NK15-142, CU03-170, CU03-171) was designed to deliver 1.5 mg 2,4-DNP/60 mg beads; however, this did not take into account a 22% moisture content in the active pharmaceutical ingredient (API), which resulted in an actual API concentration closer to 1.17 mg/60 mg beads. Subsequent batches were formulated similarly to the latter but were adjusted to deliver 1.0 mg DNP/50 mg beads (or 1.17 mg/ 58.5 mg beads). The following table shows the composition of the initial **uncoated** bead formulations.

	NK15-142, CU03-170, CU03-171					**NK15-155**				
Ingredient	**%w/w (wet)**	**g wet/ batch**	**%w/w (dry)**	**g dry/ batch**	**mg/ dose**	**%w/w (wet)**	**g wet/ batch**	**%w/w (dry)**	**g dry/ batch**	**mg/ dose**
Mannitol	65.3	391.8	65.7	391.8	39.4	65.4	394.8	65.8	394.8	32.9
MCC	32.0	192.0	32.2	192.0	19.3	31.8	192.0	32.0	192.0	16.0
HPMC	0.2	1.2	0.2	1.2	12.1	0.2	1.2	0.2	1.2	0.1
2,4-DNP[1]	2.5	15.0	2.0	11.7	1.2	2.5	15.4	2.0	12.0	1.0
Water	---	200.0[2]	---	---	---	---	200.0	---	---	---
Total	**100.0**	**600.0**	**100.0**	**596.7**	**60.0**	**100.0**	**603.4**	**100.0**	**600.0**	**50.0**

[1]2,4-DNP contains 22% moisture. The %w/w wet shows the weight percent of wet API dispensed for the batch. The dry basis %w/w and mg/dose takes into account only the solids concentration, omitting the 22% moisture in the API.
[2]Water is used as a processing aid and does not appear in the final product. It is not represented in the %w/w or final batch weight and is only included to show the quantity added for processing.

To make the uncoated spheres, the solid components were added to a high shear mixer and mixed until visually uniform (1 minute at mix speed 300 rpm). 200 ml water was then added, and the solution was mixed until visually uniform (2-3 minutes at mix

speed 300 rpm). The material was discharged and extruded with a 0.7mm die face, 9 shims, at 90 rpm. The extrudate was divided into two batches, and each was spheronized with a 2 mm plate at 980 rpm for 2 minutes. The beads were then placed in an oven and dried until the final moisture content was <7.5% based on starting weight.

Controlled Release Bead Formulation Development

In order to achieve controlled (12-24 hr) release, two polymer systems were selected for evaluation: an Eudragit RS/RL system and a hydroxypropylcellulose (HPC)/ethylcellulose (EC) system. The Eudragit system functions by combining permeable and impermeable pH independent swelling polymers, allowing the drug to diffuse out over time, controlled by both the ratio of permeable and impermeable polymer as well as amount of polymer applied. The HPC/EC system functions by combining soluble and insoluble polymers to create pores in the coating through which the drug diffuses over time, also controlled by the ratio of the polymers and the amount of polymer applied. Formulations for the two coating suspensions are described in the following table.

	NK15-146 (Eudragit System)				**NK15-143 (HPC/EC System)**			
Ingredient	**%w/w**	**% solids**	**g solids/ batch**	**g/ batch**	**%w/w**	**% solids**	**g solids/ batch**	**g/ batch**
Eudragit RS 30 D	42.0	30.0	168.0	560.0	---	---	---	---
Eudragit RL 30 D	10.5	30.0	42.0	140.0	---	---	---	---
ECN10	---	---	---	---	6.92	100.0	103.8	103.8
Klucel EF	---	---	---	---	0.77	100.0	11.5	11.5
Plasacryl T20	7.8	20.0	20.8	104.0	---	---	---	---
TEC	2.4	100.0	32.0	32.0	---	---	---	---
Polysorbate 80	0.9	33.0	4.0	12.0	---	---	---	---
Talc	---	---	---	---	1.54	100.0	23.1	23.1
DBS	---	---	---	---	0.77	100.0	11.5	11.5
DI Water	36.4	0.0	0.0	485.3	9.00	0.0	0.0	135.0
Ethanol	---	---	---	---	81.00	0.0	0.0	1215.0
Total	**100.0**	**20.0**	**266.8**	**1333.3**	**100.00**	**10.0%**	**100.0**	**1500.0**

To generate the controlled-release coating with the Eudragit system, the Plasacryl was shaken and mixed. While mixing, the Eudragits, water, TEC, and PS80 were added. The suspension was then passed through a 30 mesh screen and mixing was continued. To coat the DNP spheres produced as described above, the FLM1 fluid bed was set up with bottom spray Wurster coating with a 1.2 mm liquid nozzle, 3 mm air cap, Wurster column, 400 mesh inlet screen, conidor plate, and 40 mesh filters. The spheres were loaded into the fluid bed and coated using the following parameters:

Parameter	**Target range**
Spray rate	15-25 g/min
Inlet temperature	44-47°C
Product temperature	34-37°C
Nozzle air	40 psi
Process air	55-70 cfm

The beads were then dried for a minimum of 10 min with an inlet temperature of 40°C.

Two lots of beads coated with the HPC/EC system were successfully coated and sampled at three theoretical weight gains (11.7%, 13.3%, and 15% for Lot NK15-144 and 7.5%, 10%, and 11.7% for Lot NK15-157). The final coated bead formulation for each lot (based on a 1 mg dose and the maximum coating applied) is described below.

Lot #	**NK 15-144 (15.0% weight gain)**		**NK 15-157 (11.7% weight gain)**	
Ingredient	**% w/w**	**mg/dose**	**% w/w**	**mg/dose**
2,4-DNP drug spheres	87.0	51.3	89.5	50.0
ECN 10	9.0	5.3	7.3	4.1
Klucel EF	1.0	0.6	0.8	0.5
Talc	2.0	1.2	1.6	0.9
Dibutylsulfate	1.0	0.6	0.8	0.5
Total	**100.0**	**58.9**	**100.0**	**55.9**

Beads coated with the Eudragit system began to repeatedly block (clump) approximately 2/3 into the run and the run was ended. Due to time constraints and the

success with the HPC/EC system, process optimization for successful coating with the Eudragit system was never evaluated.

Extended release beads prepared successfully with the HPC/EC system were evaluated for assay and dissolution. The results are described below.

Lot #	% Label Claim
NK15-144-01 (11.7%)	97.4
NK15-144-12 (13.3%)	93.0
NK15-144-23 (15.0%)	94.4
NK15-157-01 (7.5%)	92.8
NK15-157-12 (10.0%)	95.9
NK15-157-23 (11.7%)	96.7

Final CRMP Formulation

A successful sustained release dosage form (beads) was developed using an HPC/EC coating system. The 12 hour targeted release profile was confirmed through in vitro dissolution testing. The final formulation representing the 2,4-DNP drug spheres is as follows:

Ingredient	%w/w	g/batch	mg/dose
Mannitol	65.8	394.8	32.9
MCC	32.0	192.0	16.0
HPMC	0.2	1.2	0.1
2,4-DNP	2.0	12.0	1.0
Total	**100.0**	**600.0**	**50.0**

These drug spheres were used to generate the controlled-release DNP drug with composition as described in the following table.

Ingredient	%w/w	mg/dose
2,4-DNP drug spheres	89.5	50.0
ECN 10	7.3	4.1
Klucel EF	0.8	0.5
Talc	1.6	0.9
DBS	0.8	0.5
Total	**100.0**	**55.9**

The first attempt to evaluate the Eudragit coating system was unsuccessful, however, with additional process development, it is likely to be successful at achieving the targeted release profile. Because of the success with the HPC/EC coating system, however, we do not plan to re-evaluate the Eudragit system at this point.

Animals

Sprague-Dawley rats (300-400g) and Zucker Diabetic Fatty rats (250g) were ordered from Charles River Laboratories and allowed to acclimate for at least one week before use. All procedures were approved by the Yale University Animal Care and Use Committee. If not otherwise specified, rats were fed regular chow. In the NAFLD reversal model, the rodents were fed a safflower oil based high fat diet (60% calories from fat) (Harlan) for two weeks before any studies were initiated. To determine whether CRMP prevents NAFLD, we fed rats safflower oil high fat diet for two weeks while treating them daily with CRMP (1 mg/kg) or vehicle. To induce NASH, we fed rats methionine/choline deficient diet (Harlan) for eight weeks, then continued them on this diet while treating them with 1 mg/kg CRMP or vehicle. Rats used in the long-term (six week) toxicity studies were treated with either peanut butter vehicle, 1, 2, or 10 mg/kg DNP, or 1, 50, or 100 mg/kg CRMP daily. Thermal algesia was assessed in these rats as described by Mo et al. (*8*)

Surgery was performed under isoflurane anesthesia to place polyethylene catheters (Instech Solomon) in the common carotid artery, jugular vein, and, where specified, antrum of the stomach (PE50, PE90, and PE90 tubing, respectively), then were allowed to recover for 1 week before any studies were performed. Animals were not

studied unless they had recovered their pre-surgery body weight. Rats were fasted for 6 hrs for measurement of DAG concentrations, and overnight (~16 hrs) for all other studies. For terminal studies, rats were euthanized by intravenous pentobarbital.

For the intragastric DNP infusion studies, rats previously fed high fat diet for 2 weeks were placed in a Covance infusion harness attached to a single-axis counter-balanced swivel mount and a stainless steel one-channel swivel (all from Instech Solomon) to protect the catheters and allow the animals free access to the cage. DNP (2 mg/kg per day) or 10% dimethyl sulfoxide/90% saline vehicle was infused continuously through the gastric line for 5 days. After they were placed in the infusion harness, the rats were not handled again during the course of the 5 day study.

Rats used for the NAFLD reversal studies were fed high fat diet for 2 weeks, at the end of which they were treated for three days with peanut butter to acclimate them to this food. They were then treated daily with CRMP mixed in peanut butter, or peanut butter vehicle, at the doses specified in the text daily for five days, with each rat receiving just one dose for the duration of the study. All rats consumed the peanut butter with or without CRMP within 2 minutes. Zucker Diabetic Fatty rats were treated with CRMP (1 mg/kg) in peanut butter or peanut butter vehicle daily for 14 days. Blood glucose was measured by glucometer (Abbott) from the tail vein every three days. Rats used for the NASH reversal studies were fed methionine/choline deficient diet for eight weeks as described above, then treated with 1 mg/kg CRMP or vehicle daily for six weeks while continuing on methionine/choline deficient diet.

Normal male C57BL/6J mice were ordered at 12 weeks of age from Jackson and were fed regular chow. After acclimating for five days, they underwent metabolic cage

(CLAMS) analysis. The reported food intake does not include caloric intake from the small amount of peanut butter (~250 mg) used to administer CRMP or vehicle; however, the peanut butter quantity was matched between groups.

Toxicity studies

Alanine aminotransferase, aspartate aminotransferase, and blood urea nitrogen were measured in rats treated with varying doses of DNP or CRMP using the COBAS Mira Plus, and creatinine was measured by LC/MS/MS. Body temperature was measured using a rectal probe (Physitemp Instruments). Histology slides were prepared by the Yale Research Histology core and were stained with hematoxylin & eosin, Sirius Red, and TUNEL stains.

Studies of basal metabolism

Plasma insulin and glucagon were measured by radioimmunoassay by the Yale Diabetes Research Core. Plasma glucose was measured enzymatically by the YSI Life Sciences 2700 Select Biochemistry Analyzer. Non-esterified fatty acid concentrations were measured by an enzymatic kit (Wako). Plasma concentrations of twelve inflammatory markers, adiponectin, FGF-21, and albumin were assessed by ELISA (QIAGEN, Life Technologies, Millipore, and Abcam, respectively). Liver glycogen was measured by amyloglucosidase digestion (*9*).

Lipid measurements

Plasma triglycerides were measured using a Wako reagent. Liver and quadriceps triacylglycerol were extracted using the method of Bligh and Dyer (*10*) and quantified spectrophotometrically using a reagent from Diagnostic Chemicals. Liver and quadriceps DAG, ceramide, and acylcarnitine concentrations were measured by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) (*11*). Very low-density lipoprotein (VLDL) export was measured as Lee et al. have described (*12*). Liver acetyl CoA was measured as previously reported (*13*).

Markers of liver fibrosis and apoptosis

Caspase-3 and -9, smooth muscle actin, and inflammatory cytokines were measured in liver homogenates by ELISA (MyBioSource, NeoBioLab, MyBioSource, and SABiosciences, respectively), and were normalized to total protein content measured by Bradford assay. Liver hydroxyproline content was measured as described previously (*14*). Collagen mRNA was measured in liver, and UCP1 mRNA in BAT by qPCR (*15*).

Glucose tolerance tests

Rats were given an intraperitoneal bolus of 50% dextrose (1 mg/kg) at time zero. Plasma was obtained by drawing blood through the venous catheter at times 0, 5, 10, 20, 30, 45, 60, and 90 min and centrifuged immediately. Plasma glucose and insulin concentrations were measured as described above.

Measurement of insulin sensitivity and hepatic fluxes

Basal and insulin-stimulated glucose turnover were measured using a steady-state (120 min) infusion of [6,6-$^{2}H_2$] glucose (*16*). Muscle and BAT glucose uptake was measured by injection of [^{14}C]2-deoxyglucose into the venous line as previously reported (*17*). PKCε and PKCθ translocation in liver and quadriceps, respectively, were measured by Western blot as previously described (*16*). Liver flux through the TCA cycle and substrate contributions to the TCA cycle were measured in liver, kidney, heart, skeletal muscle, and brain by steady-state [3-^{13}C] lactate infusion as we have described (*4*), with the exception of the calculation of the percent gluconeogenesis from pyruvate, which was measured using the following formula:

$$\frac{V_{GNG\ pyrvuate}}{V_{GNG}} = \frac{^{13}C_{1,2,3,4,5,6}\text{glucose} + {}^{13}C_{3}\,\text{glucose} + {}^{13}C_{4}\text{glucose}}{2 * ({}^{13}C_{1,2,3,4}\text{malate})}.$$

Measurement of hepatic protein concentrations

Hepatic pyruvate carboxylase, glucose-6-phosphatase, and PEPCK protein concentrations were measured using antibodies from Santa Cruz Biotechnology and CD69 was measured using an antibody from Novus Biologicals, as we have reported (*14*). PKC translocation was measured as previously reported (*17*).

DNP and CRMP kinetics studies

Rats consumed the concentrations of DNP or CRMP specified in the text orally, mixed in peanut butter. All rats used for the kinetics studies consumed the entirety of the peanut

butter and protonophore within 2 minutes. DNP concentrations were measured in plasma, liver, kidney, WAT, quadriceps, heart, and brain of Sprague-Dawley rats by LC/MS/MS as previously described *(8)*. Rats treated with DNP were sacrificed 1 hour after treatment with DNP, and those treated with CRMP were sacrificed 8 hours after treatment, as these were the times determined in the plasma DNP studies to represent the peak plasma DNP concentrations (Fig. S4A-B). The minimal detection limit of our method was determined to be 0.05 μM, the minimum concentration of solutions of known quantities of DNP in DMSO measured by our method with less than 20% error in each of three replicates.

Statistical analysis

Differences were assessed by the 2-tailed unpaired Student's t-test when two groups were compared, or by ANOVA with Bonferroni's multiple comparisons test when three groups were compared. P-values < 0.05 were considered significant. Data are reported as mean ± S.E.M.

Figure S1

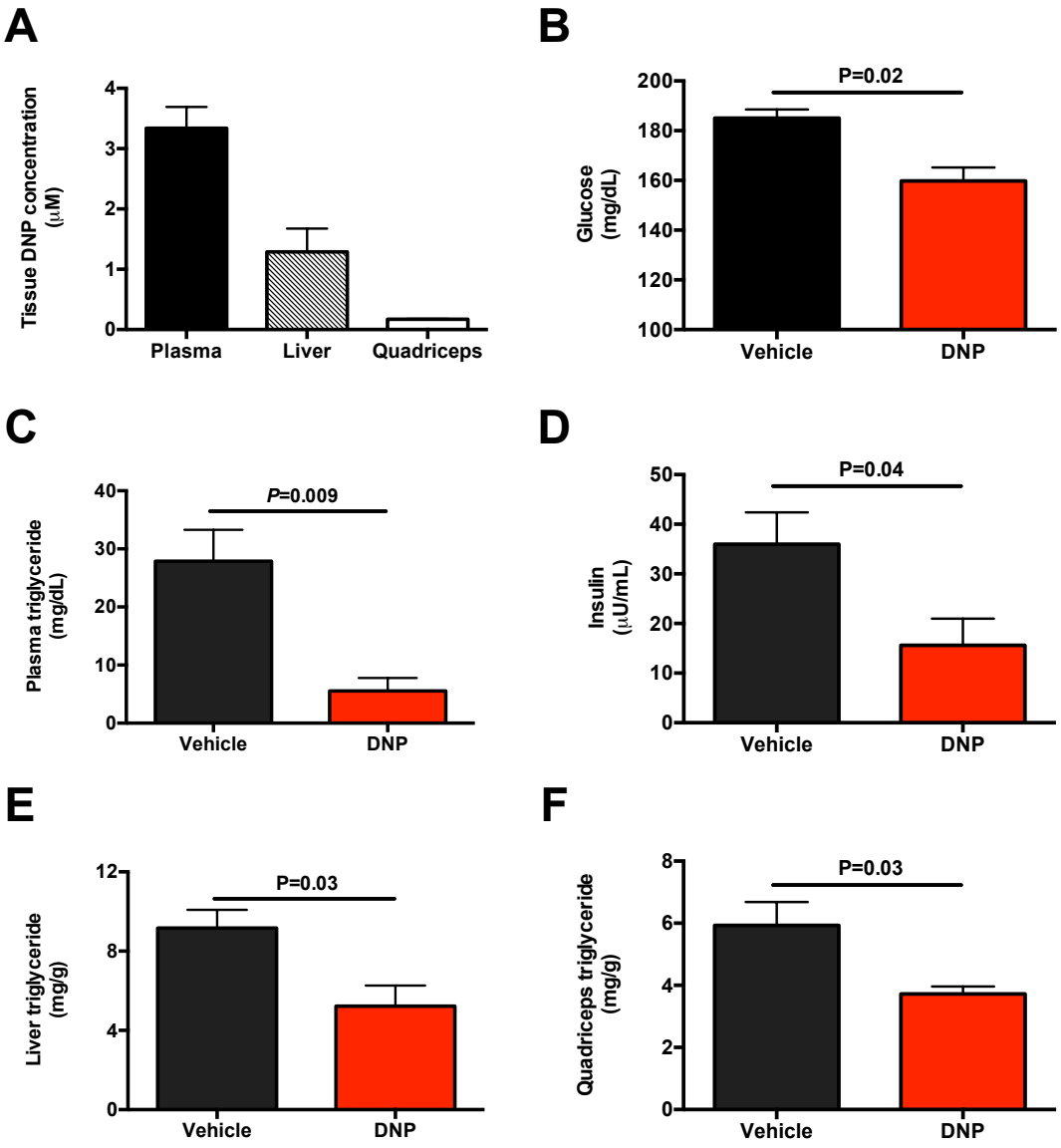

Figure 1. Continuous intragastric DNP infusion safely reverses hypertriglyceridemia, NAFLD and insulin resistance in the rat. **(A)** Tissue DNP concentrations. **(B)-(D)** Fasting plasma glucose, triglyceride and insulin concentrations. **(E), (F)** Liver and quadriceps triglyceride. Data are mean ± S.E.M. of n=4 per group, with comparisons by t-test.

Figure S2

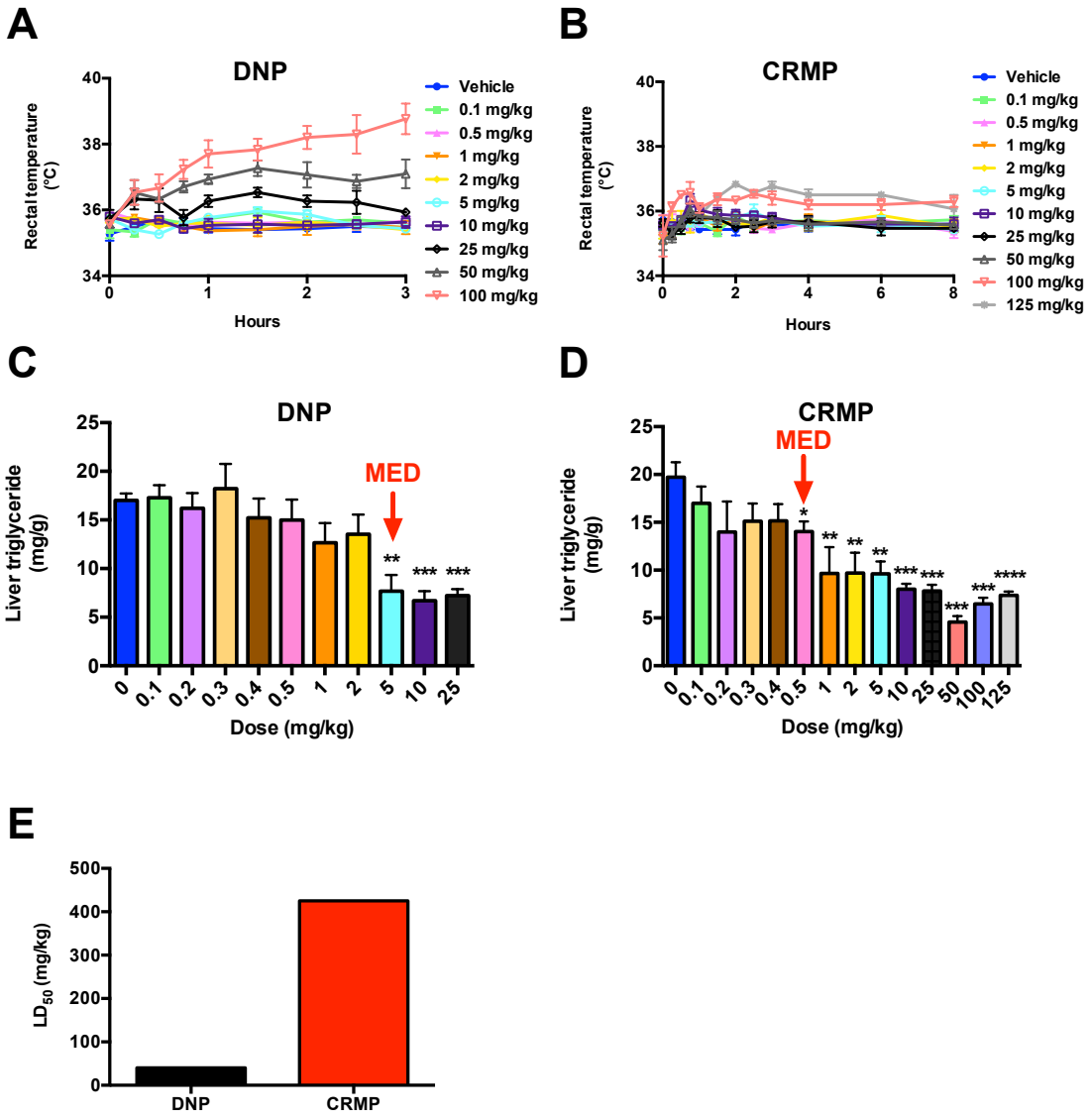

Figure S2. CRMP is safe and effective at reversing NALFD in rats. **(A), (B)** Rectal temperature after varying doses of DNP **(A)** or CRMP **(B)** administered at time 0. **(C), (D)** Hepatic triglyceride content after two weeks of high fat diet, and an additional 2 week treatment period with varying doses of DNP **(C)** or CRMP **(D)**. **(E)** LD_{50} of DNP and CRMP. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. vehicle-treated rats by t-test. In all panels, data are mean ± S.E.M. of n=3-4 per group.

Figure S3

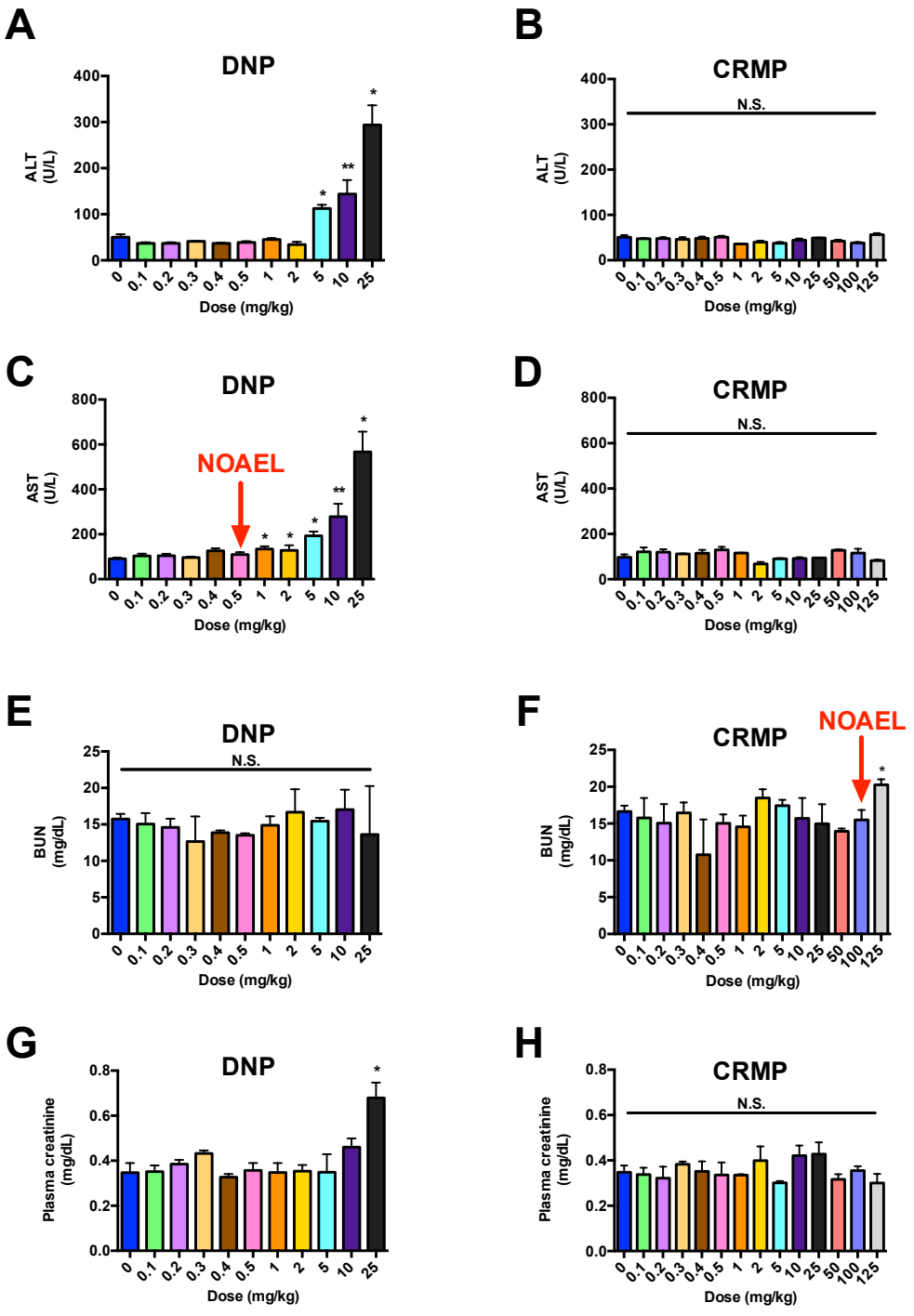

Figure S3. The five-day no observed adverse effect level (NOAEL) of CRMP is 200-fold higher than DNP. **(A)**, **(B)** ALT. **(C), (D)** AST. **(E), (F)** BUN**. (G), (H)** Creatinine. In all panels, data are mean ± S.E.M. of n=3-4 per group. **P*<0.05, ***P*<0.01 vs. vehicle by t-test.

Figure S4

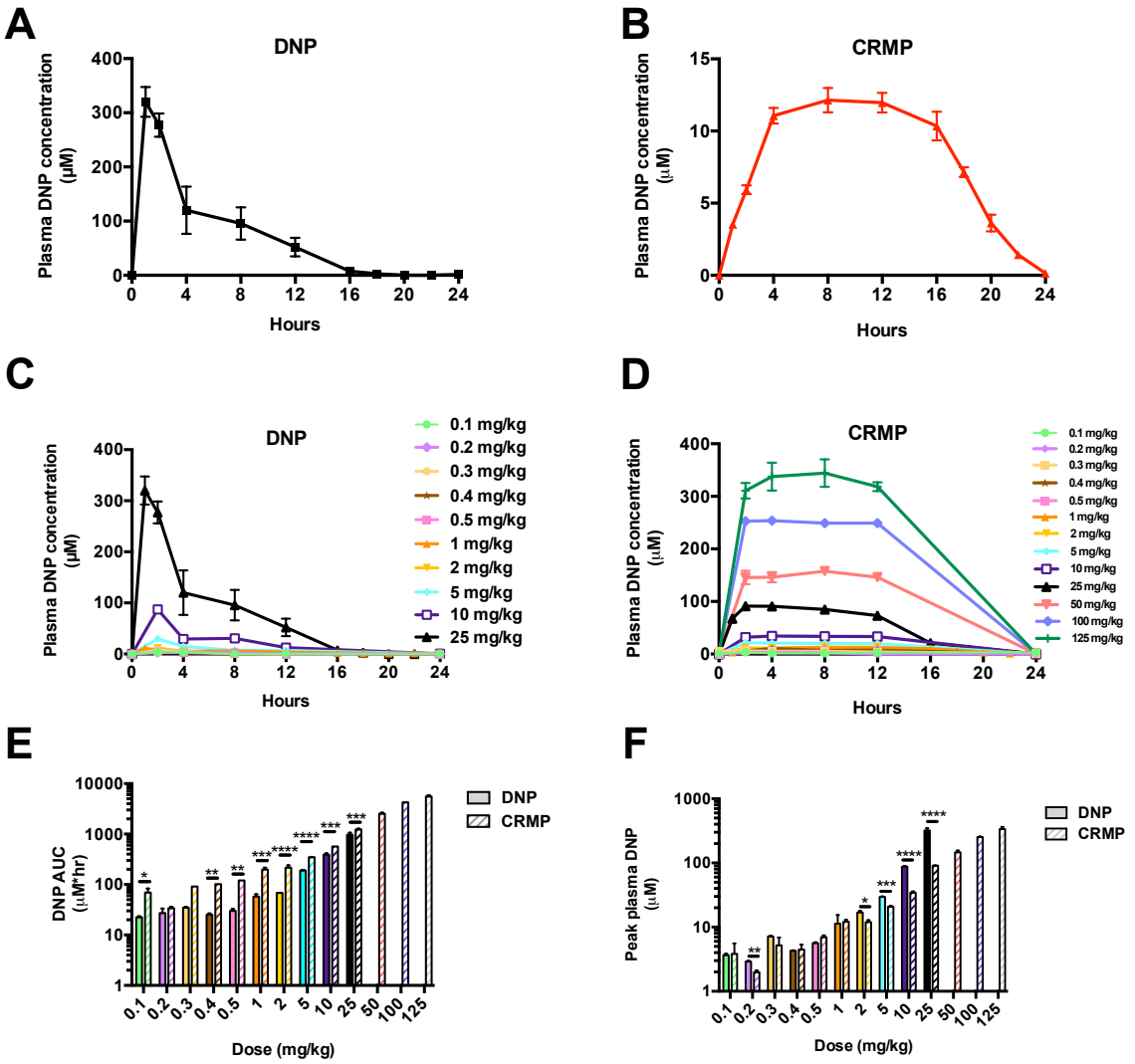

Figure S4. CRMP treatment results in lower peak plasma DNP concentrations and higher DNP areas under the curve than DNP treatment. **(A)** Plasma DNP concentrations after treatment with 25 mg/kg DNP (toxic dose). These data are repeated in panel **(C)**. n=6 per group. **(B)** Plasma DNP concentrations after treatment with 1 mg/kg CRMP (effective dose). These data are repeated in panel **(D)**. n=9 per group. **(C), (D)** Plasma DNP concentrations after varying doses of DNP **(C)** or CRMP **(D)**. **(E)** Plasma DNP area under the curve for treatment with varying doses of DNP or CRMP. **(F)** Peak plasma DNP concentrations after treatment with varying doses of DNP or CRMP. Data are mean ± S.E.M. Unless otherwise specified, n=3 per group. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 by t-test.

Figure S5

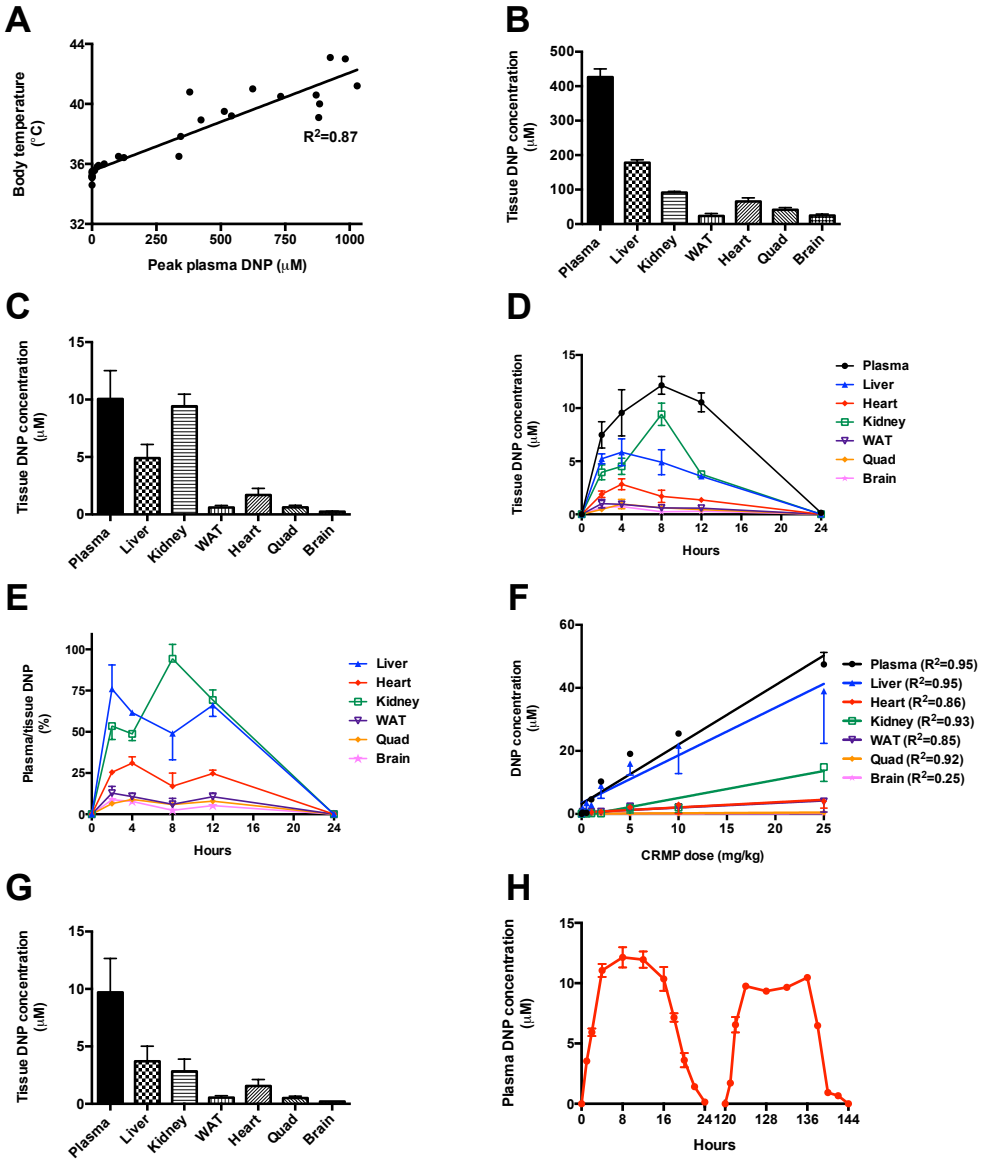

Figure S5. CRMP is better tolerated than DNP because it generates lower plasma and tissue DNP concentrations. **(A)** Correlation between rectal temperature and peak plasma DNP concentration after treatment with DNP or CRMP. **(B)** Tissue DNP concentrations 1 hr after one treatment with 25 mg/kg DNP. **(C)** Tissue DNP concentrations 8 hr after a single treatment with 1 mg/kg CRMP. **(D)** Plasma and tissue DNP concentrations at various time points after treatment with 1 mg/kg CRMP. **(E)** Plasma:tissue DNP ratio at various time points following 1 mg/kg oral CRMP. **(F)** Plasma and tissue DNP concentrations 8 hours after treatment as a function of CRMP dose. **(G)** Tissue DNP concentrations 8 hours after the last of five daily 1 mg/kg CRMP doses. **(H)** Plasma DNP concentrations after one dose and the last of five daily 1 mg/kg CRMP doses. Data are mean ± S.E.M. Unless otherwise specified, n=4.

Figure S6

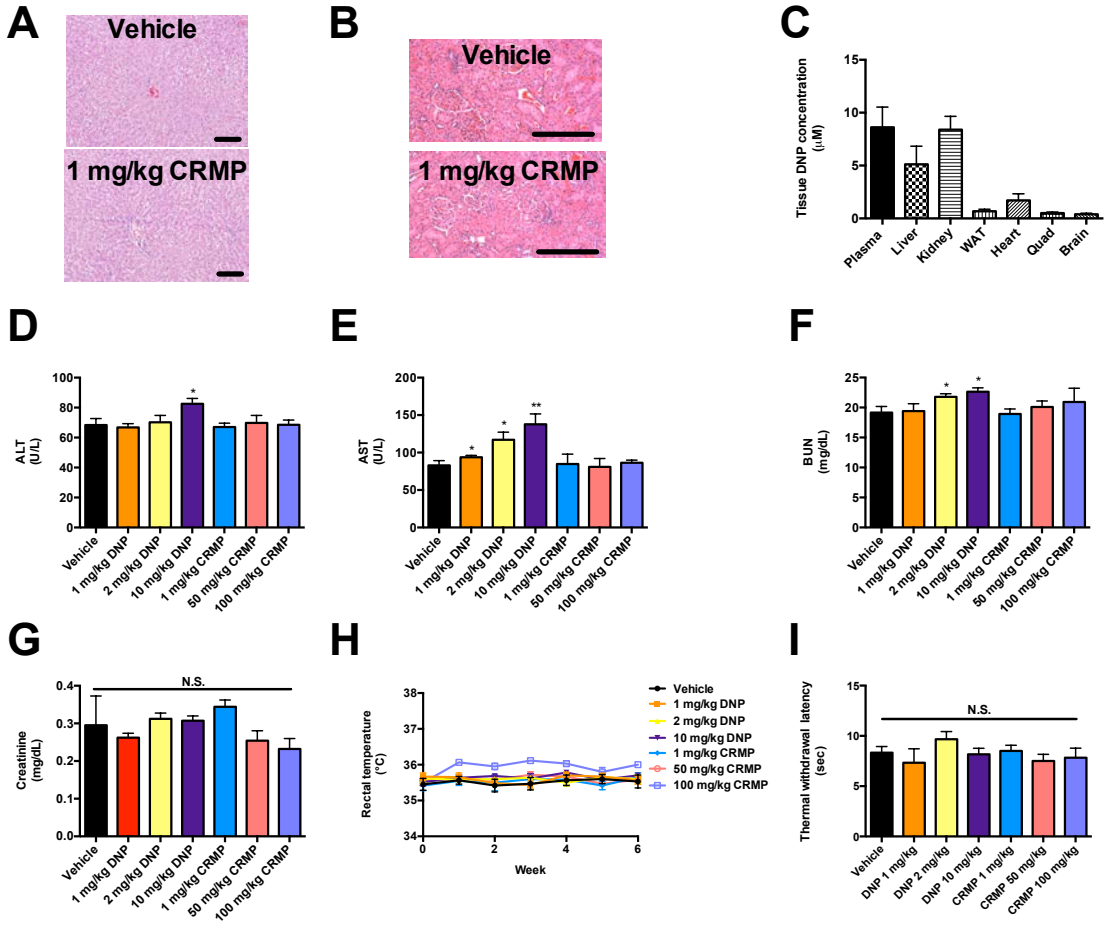

Figure S6. 6 weeks of CRMP treatment is well tolerated. **(A), (B)** Liver and kidney histology after 6 wk 1 mg/kg CRMP treatment. **(C)** Tissue DNP concentrations after 6 wk 1 mg/kg CRMP treatment. **(D)-(G)** ALT, AST, BUN and creatinine concentrations. **(E), (F)** Liver and kidney histology. Scale bars, 100 μm**. (H)** Rectal temperature. **(I)** Thermal algesia. In all panels, data are mean ± S.E.M. of n=6 per group. In panels **(D)-(I)**, **P*<0.05, ***P*<0.01 versus vehicle-treated rats by t-test.

Figure S7

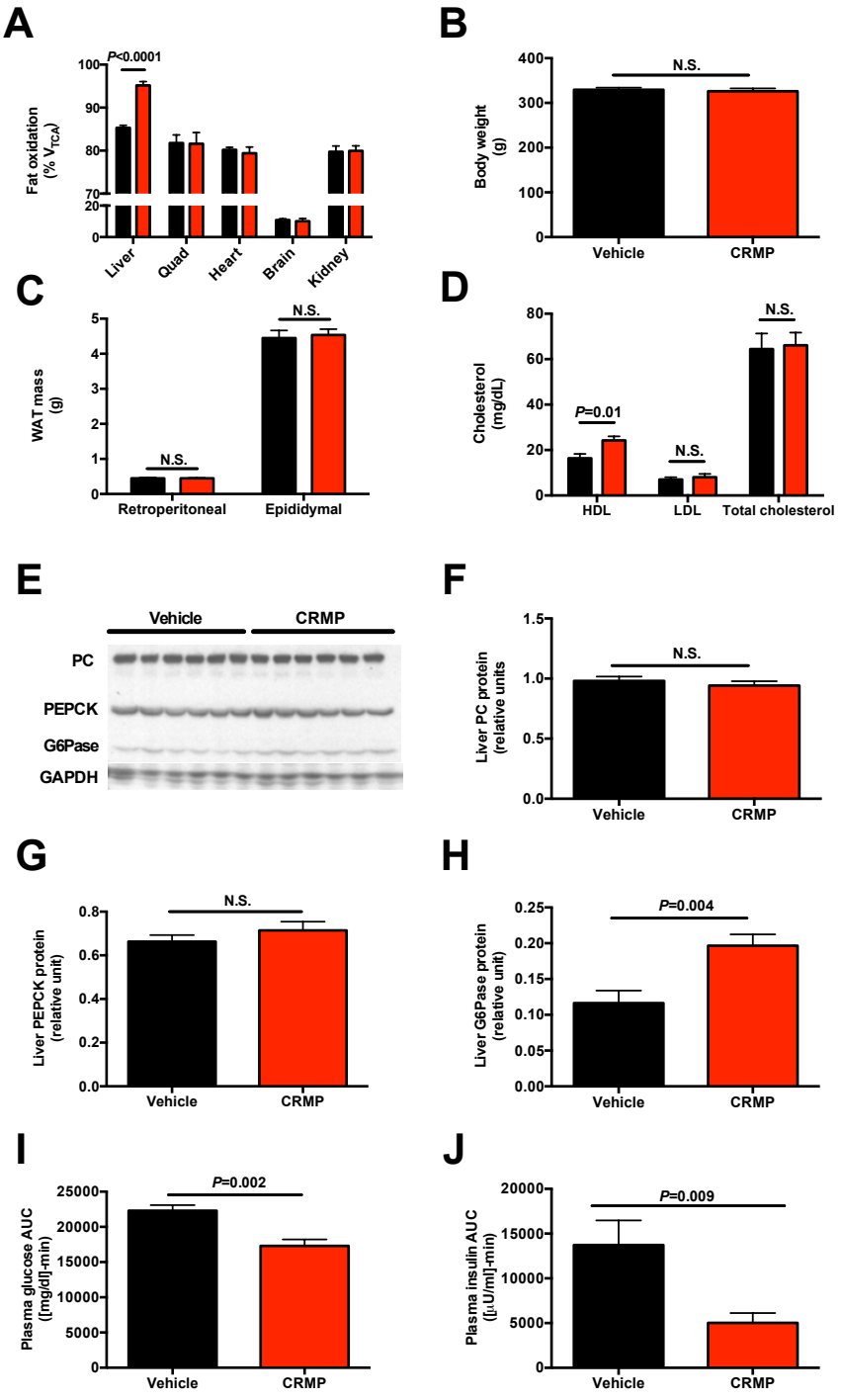

Figure S7. CRMP treatment improves hepatic insulin sensitivity in high fat-fed rats. **(A)** Hepatic fat oxidation, expressed as a percentage of total V_{TCA} flux. **(B)** Body weight. **(C)** Fat pad mass. **(D)** Plasma cholesterol. **(E)-(H)** Liver gluconeogenic protein expression. **(I)-(J)** Plasma glucose and insulin area under the curve in a glucose tolerance test. In all panels, data are mean ± S.E.M. of n=6-8 per group, with comparisons by t-test.

Figure S8

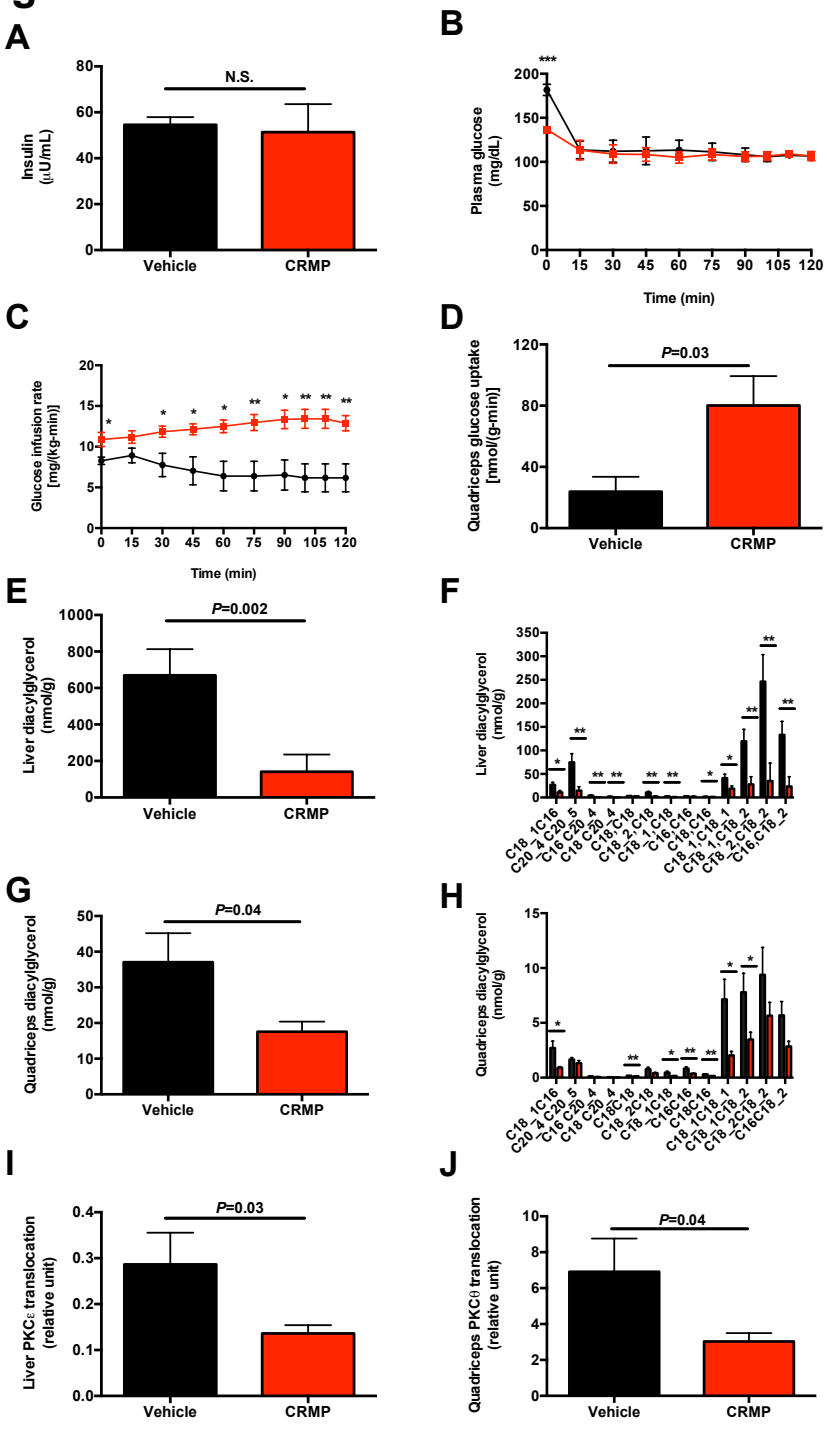

Figure S8. CRMP ameliorates NAFLD and improves insulin sensitivity by hepatic mitochondrial uncoupling. **(A)** Plasma insulin at the end of a hyperinsulinemic-euglycemic clamp. **(B), (C)** Plasma glucose and glucose infusion rate during the clamp. **(D)** Insulin-stimulated glucose uptake in quadriceps. **(E)-(H)** Liver and quadriceps DAG content. **(I), (J)** PKC translocation in liver and quadriceps. In all panels, **P*<0.05, ***P*<0.01, ****P*<0.001 by t-test. Data are mean ± S.E.M. of n=6-8 per group.

Figure S9

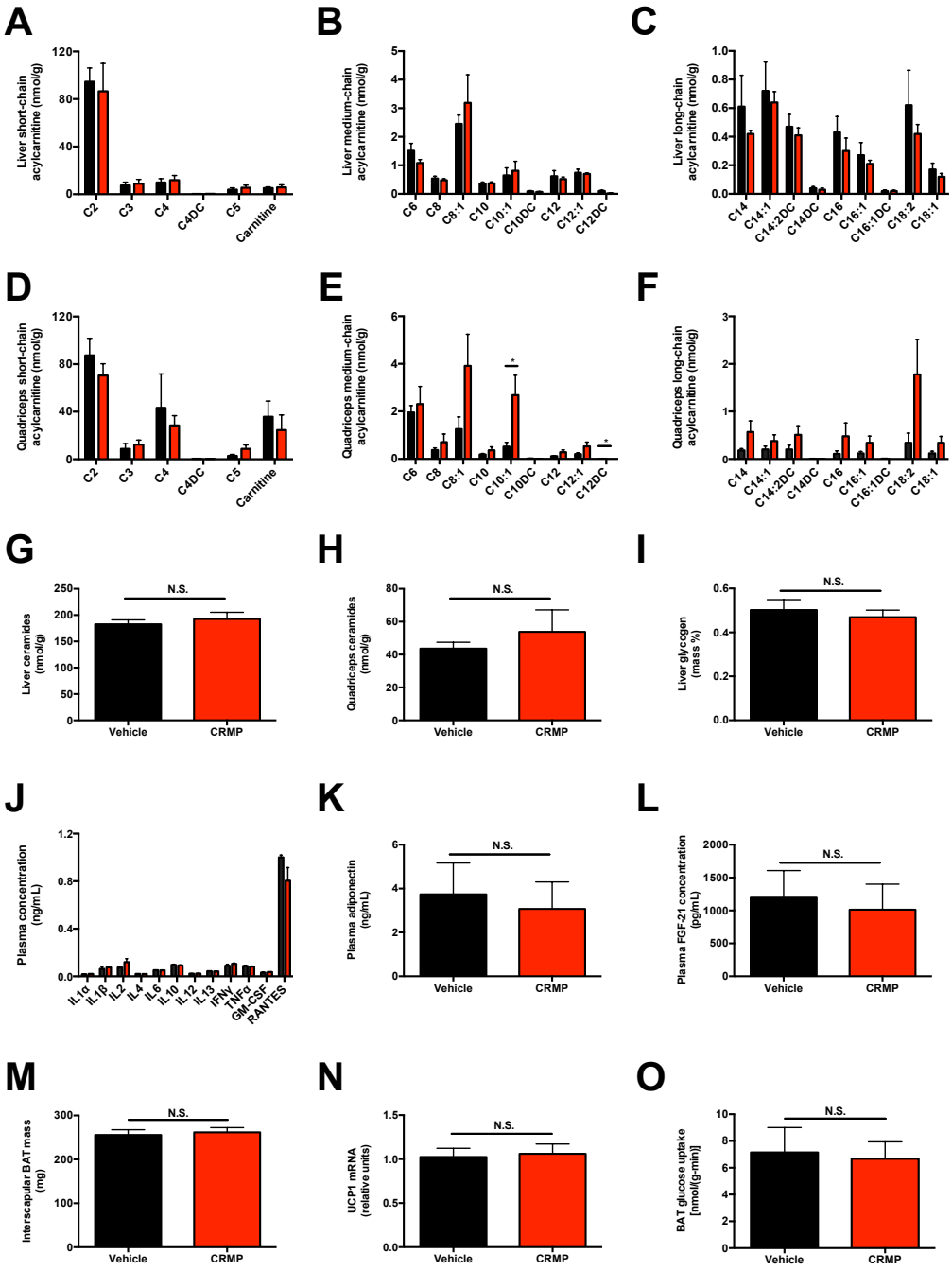

Figure S9. CRMP treatment ameliorates NAFLD and improves insulin sensitivity. **(A)-(C)** Liver acylcarnitines. In panels **(A)-(F)**, black bars, vehicle; red bars, CRMP. **(D)-(F)** Quadriceps acylcarnitines. **P*<0.05. **(G), (H)** Liver and quadriceps ceramides. **(I)** Liver glycogen. **(J)** Plasma inflammatory cytokine concentrations. **(K), (L)** Plasma adiponectin and FGF-21 concentrations. **(M)** Intrascapular brown adipose tissue mass. **(N)** BAT UCP1 mRNA expression normalized to actin. **(O)** Insulin-stimulated glucose uptake in brown adipose tissue. In all panels, data are mean ± S.E.M. of n=6-8 per group, with comparisons by t-test.

Figure S10

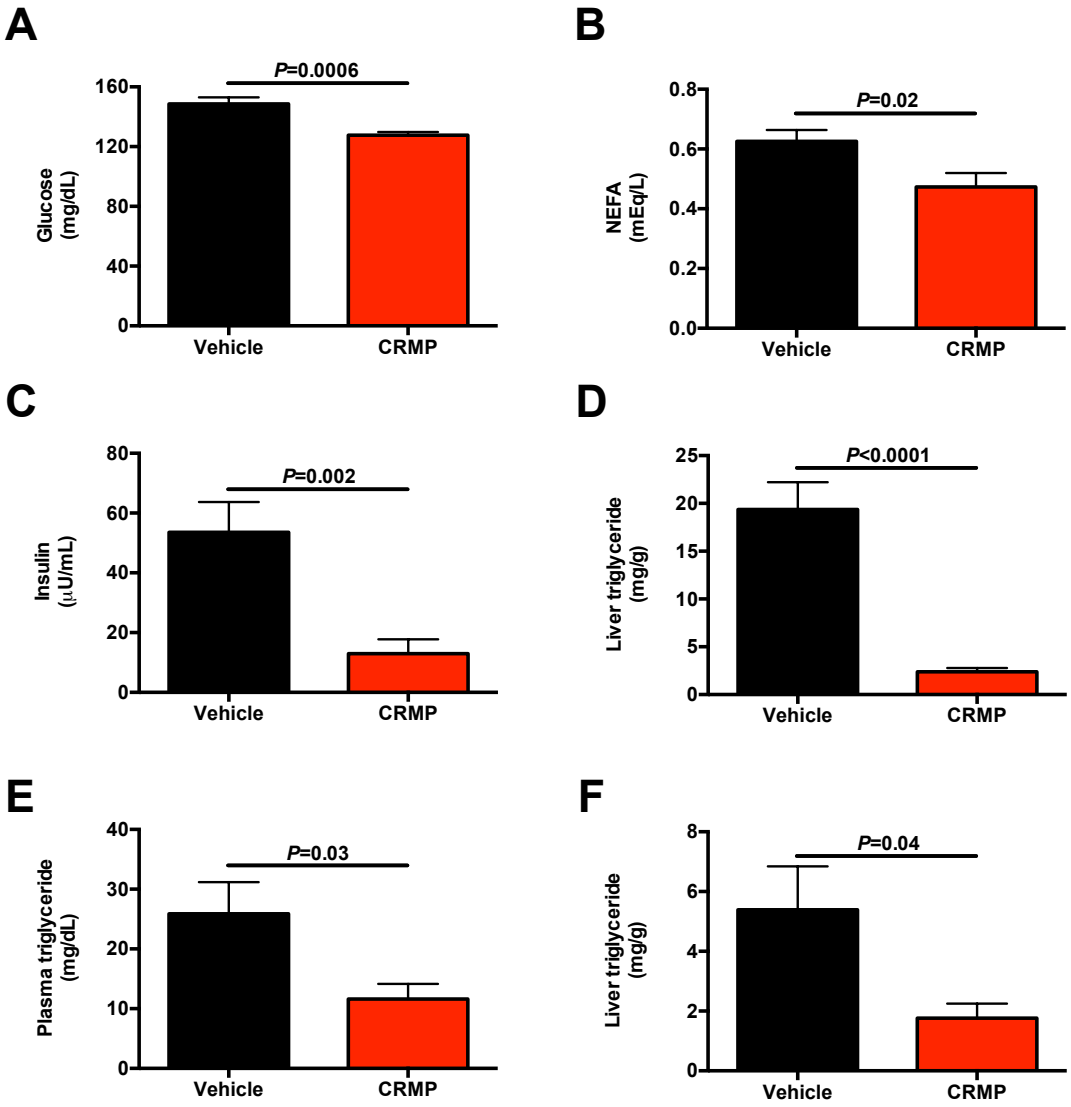

Figure S10. CRMP treatment prevented NAFLD and insulin resistance in high fat-fed rats. **(A)-(C)** Fasting plasma glucose, NEFA and insulin concentrations. **(D)-(F)** Liver, plasma, and quadriceps triglyceride content. Data are mean ± S.E.M. of n=8 per group, with comparisons by t-test.

Figure S11

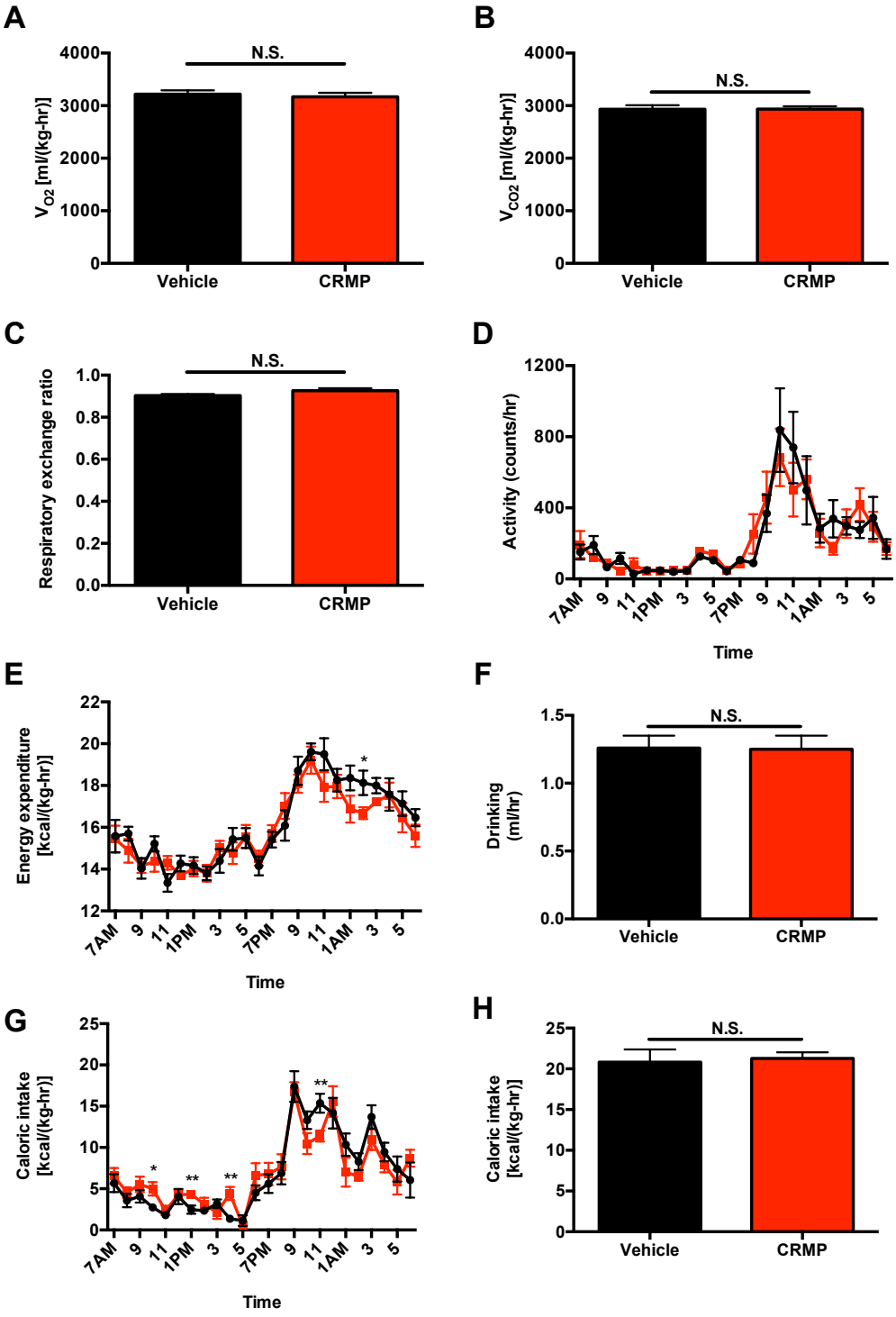

Figure S11. CRMP did not alter whole-body metabolism in mice. **(A)** Oxygen consumption (V_{O2}). **(B)** Carbon dioxide production (V_{CO2}). **(C)** Respiratory quotient. **(D)** Activity over the course of the day. **(E)** Energy expenditure throughout the day. **(F)** Total daily water drinking. **(G)** Total daily food intake. **(H)** Food intake over the course of the day. Data are mean ± S.E.M. of n=8 per group, with comparisons by t-test.

Figure S12

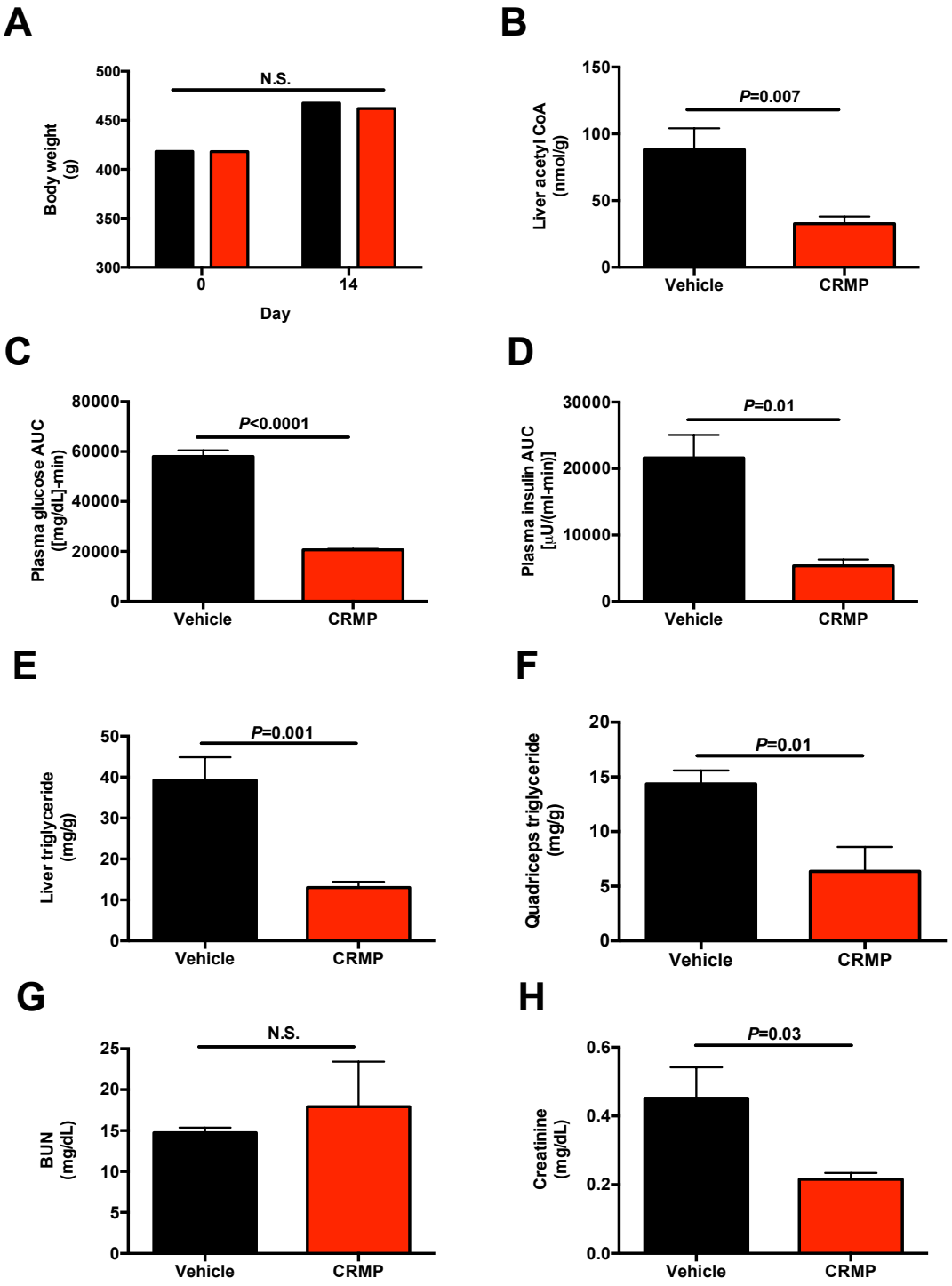

Figure S12. CRMP reverses type 2 diabetes in Zucker Diabetic Fatty rats. **(A)** Body weight before and after vehicle (black bars) or CRMP (red bars) treatment. **(B)** Hepatic acetyl CoA concentration. **(C)**, **(D)** Plasma glucose and insulin area under the curve in an IP glucose tolerance test. **(E)**, **(F)** Liver and quadriceps TAG. **(G)**, **(H)** BUN and creatinine. Data are mean ± S.E.M. of n=6-7 per group, with comparisons by t-test.

Figure S13

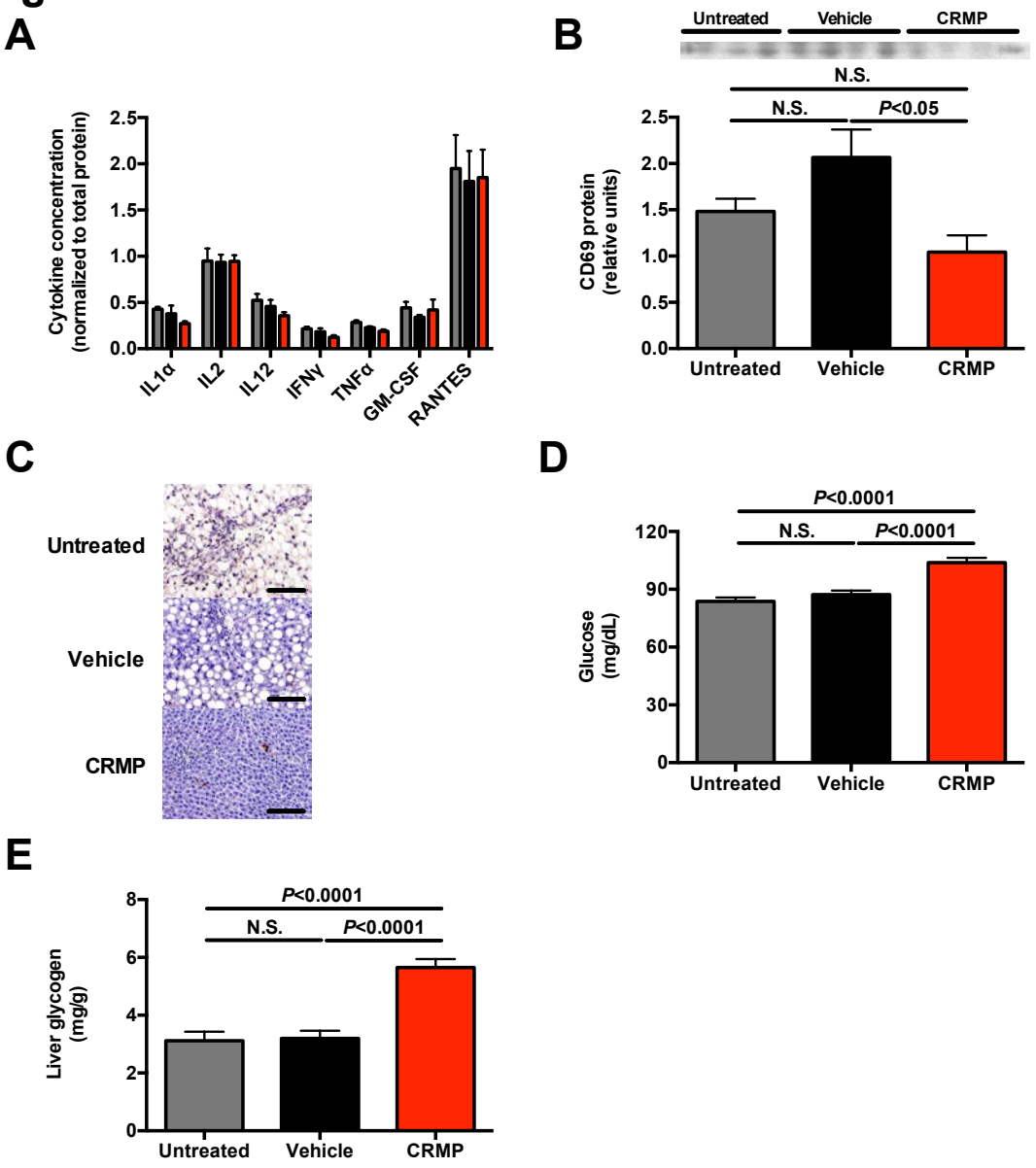

Figure S13. CRMP treatment ameliorates NASH in the rat. **(A)** Liver inflammatory cytokine protein content, normalized to total protein. **(B)** Hepatic CD69 protein. **(C)** Livers stained for TUNEL positive cells (brown stain). Scale bars, 50 μm. **(D)** Fasting plasma glucose concentrations. **(E)** Liver glycogen content. Data are mean ± S.E.M. of n=6-8 per group, with statistical significance determined by ANOVA.