Chronic high dose zinc supplementation induces visceral adipose tissue hypertrophy without altering body weight in mice

Supplementary Materials

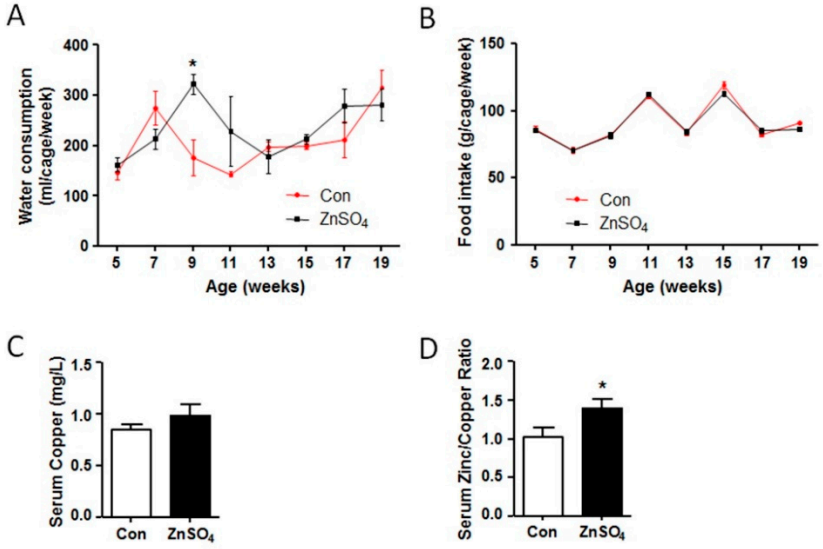

Figure S1. Water consumption and food intake of mice during the experiment. 12 mice were housed in 3 cages at 4 mice per cage. At the beginning of the week, the initial food weight and water volume for every cage were recorded. And then, the left food weight and water volume were measured at the end of the week. Food intake and water consumption were calculated as: initial food weight or water volume-left food weight or water volume. (**A**) Water consumption (P_{zinc}=0.1960; P_{time}=0.0004; $P_{zinc\ x\ time}$=0.0290) (N=3 for each group); (**B**) Food intake (P_{zinc}=0.5815; P_{time}<0.0001; $P_{zinc\ x\ time}$=0.0918) (N=3 for each group). (**C**) Serum copper levels at harvest (N=3 for each group). (**D**) Serum zinc/copper ratio at harvest (P=0.0462) (N=3 for each group). Data were shown as mean ± SEM. Con, control group; $ZnSO_4$, zinc sulfate-supplemented group. * P<0.05 $ZnSO_4$ vs. Con.

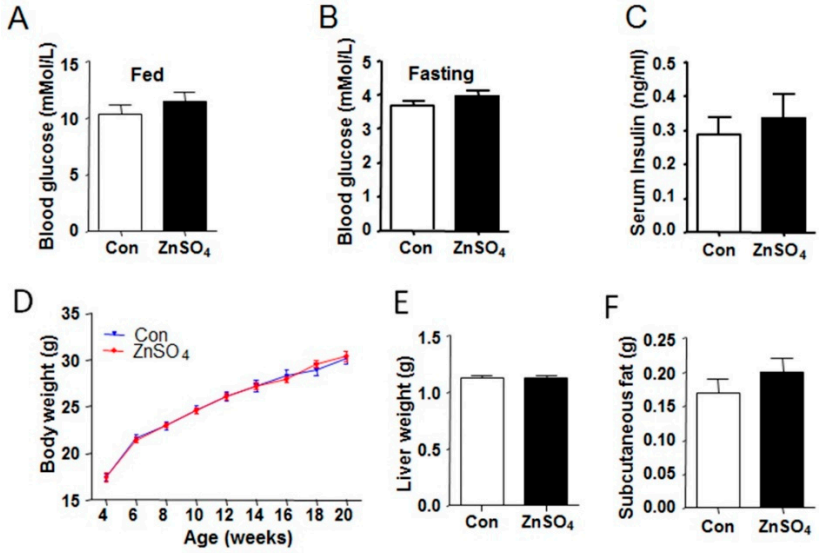

Figure S2. Blood glucose levels, body weight and tissue weight (N=8 for each group). (**A**) Blood glucose levels at fed state (N=8 for each group); (**B**) Blood glucose levels at fasting state (N=8 for each group); (**C**) Serum insulin levels (N=8 for each group); (**D**) Body weight during the experiment (N=12 for each group); (**E**) Liver weight

(N=8 for each group); (F) The weight of subcutaneous adipose tissue (N=8 for each group). Data were shown as mean ± SEM. Con, control group; $ZnSO_4$, zinc sulfate-supplemented group.

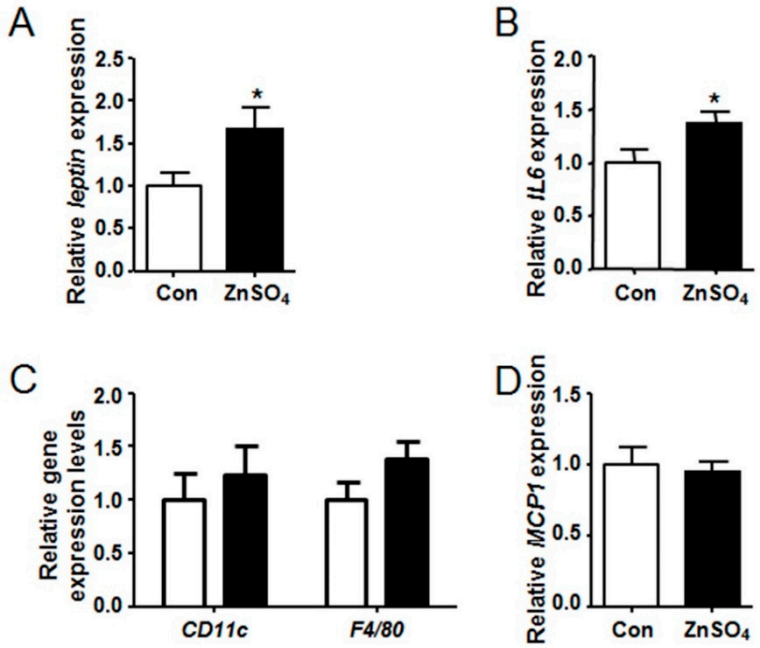

Figure S3. Gene expression in epididymal adipose tissue. (**A**) The expression of *leptin* (*P*=0.0328); (**B**) The expression of *IL6* (*P*=0.0482); (**C**) The expression of macrophage marker genes; (**D**) The expression of *MCP1*. N=8 for each group. Data were shown as mean ± SEM. Con, control group; $ZnSO_4$, zinc sulfate-supplemented group. * *P*<0.05 ZnSO4 vs. Con.

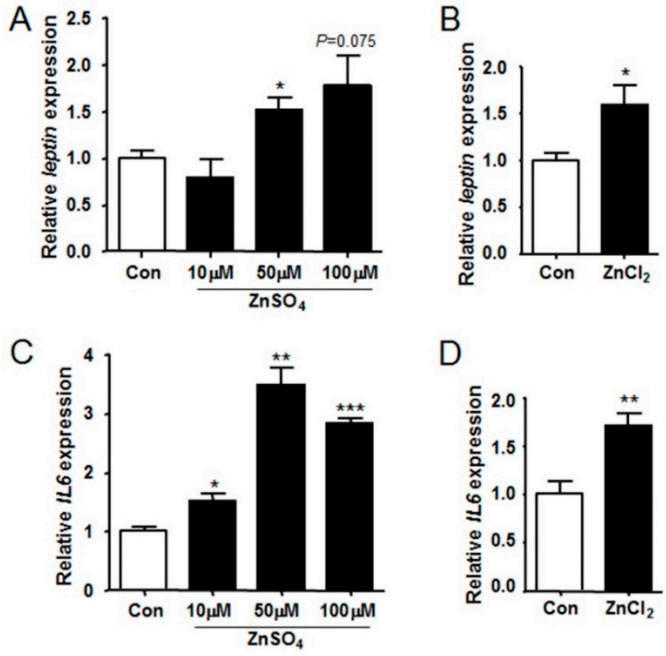

Figure S4. Gene expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated in serum-free DMEM medium for 14 h. Cells were then treated with 50 μM zinc or the vehicle for 6 h. (**A**) The expression of *leptin* after zinc sulfate treatment (P_{zinc}=0.0271, $P_{50\mu M}$=0.0238); (**B**) The expression of *leptin* after zinc chloride treatment (*P*=0.0001); (**C**) The expression of *IL6* after zinc sulfate treatment (P_{zinc}<0.0001, $P_{10\mu M}$=0.0250, $P_{50\mu M}$=0.0013, $P_{100\mu M}$=0.0001); (**D**) The expression of *IL6* after zinc chloride treatment (*P*=0.0001). N=3 for each treatment. Data

were shown as mean ± SEM. Results represented one of three repeated experiments. Con, control group; $ZnSO_4$, zinc sulfate-treated group; $ZnCl_2$, zinc chloride-treated group. * P<0.05, ** P<0.01, *** P<0.001 Zinc vs. Con.

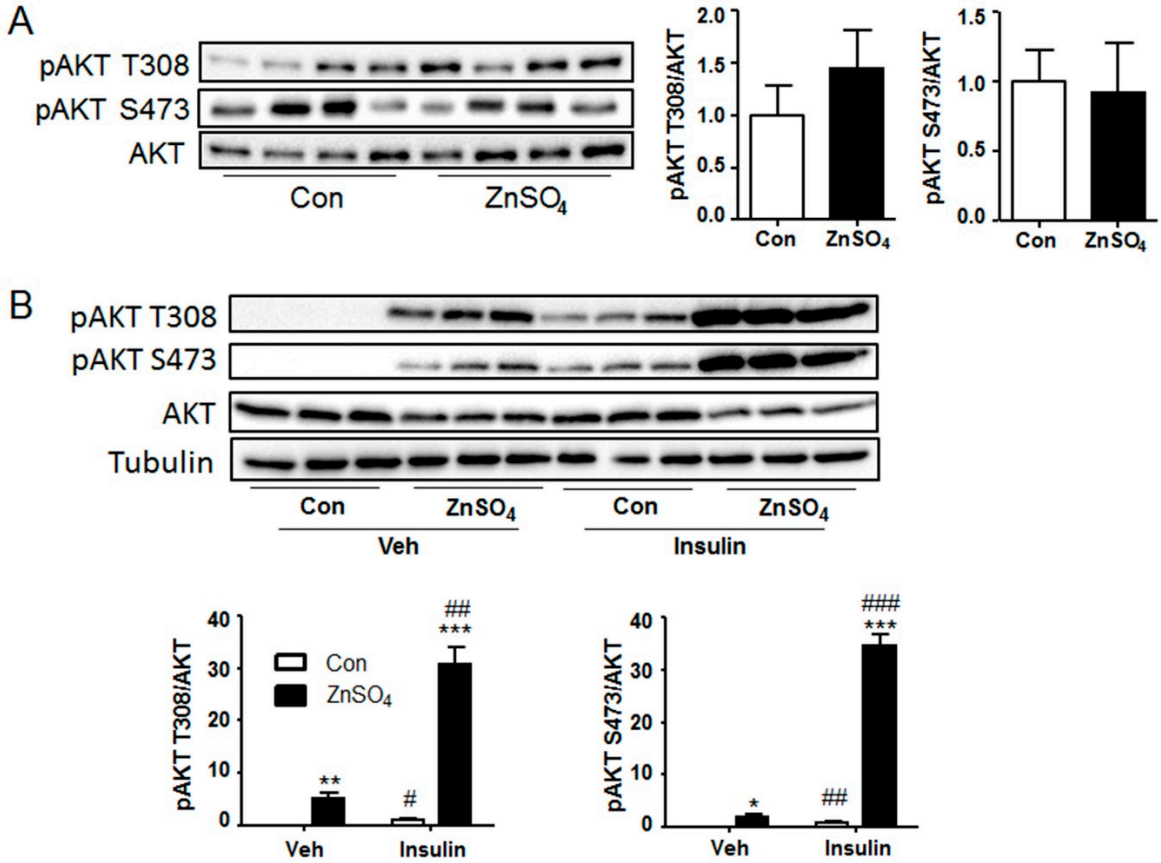

Figure S5. Phosphorylation level of AKT in epididymal adipose tissue and 3T3-L1 adipocytes. (**A**) Phosphorylation levels of AKT T308 and S473 in epididymal adipose tissue (N=8 for each group). Western blot bands represented 8 mice of 16 in total. (**B**) Phosphorylation levels of AKT T308 and S473 in 3T3-L1 adipocytes (N=3 for each group) (P_{zinc}<0.0001, $P_{Insulin}$<0.0001, $P_{Interaction}$=0.0001 for pAKT T308 and P_{zinc}<0.0001, $P_{Insulin}$<0.0001, $P_{Interaction}$<0.0001 for pAKT S473). 3T3-L1 adipocytes were incubated in serum-free DMEM medium for 14 h, followed by 6 h treatment with zinc sulfate or control. Cells were then treated with zinc sulfate or control plus 100 nM insulin for 5 min. Results represented one of three repeated experiments. Data were shown as mean ± SEM. Con, control mice or cells; $ZnSO_4$, zinc sulfate-treated mice or cells; Veh, the vehicle for insulin. * P<0.05, ** P<0.01, *** P<0.001 $ZnSO_4$ vs. Con; # P<0.05, ## P<0.01, ### P<0.001 $ZnSO_4$.

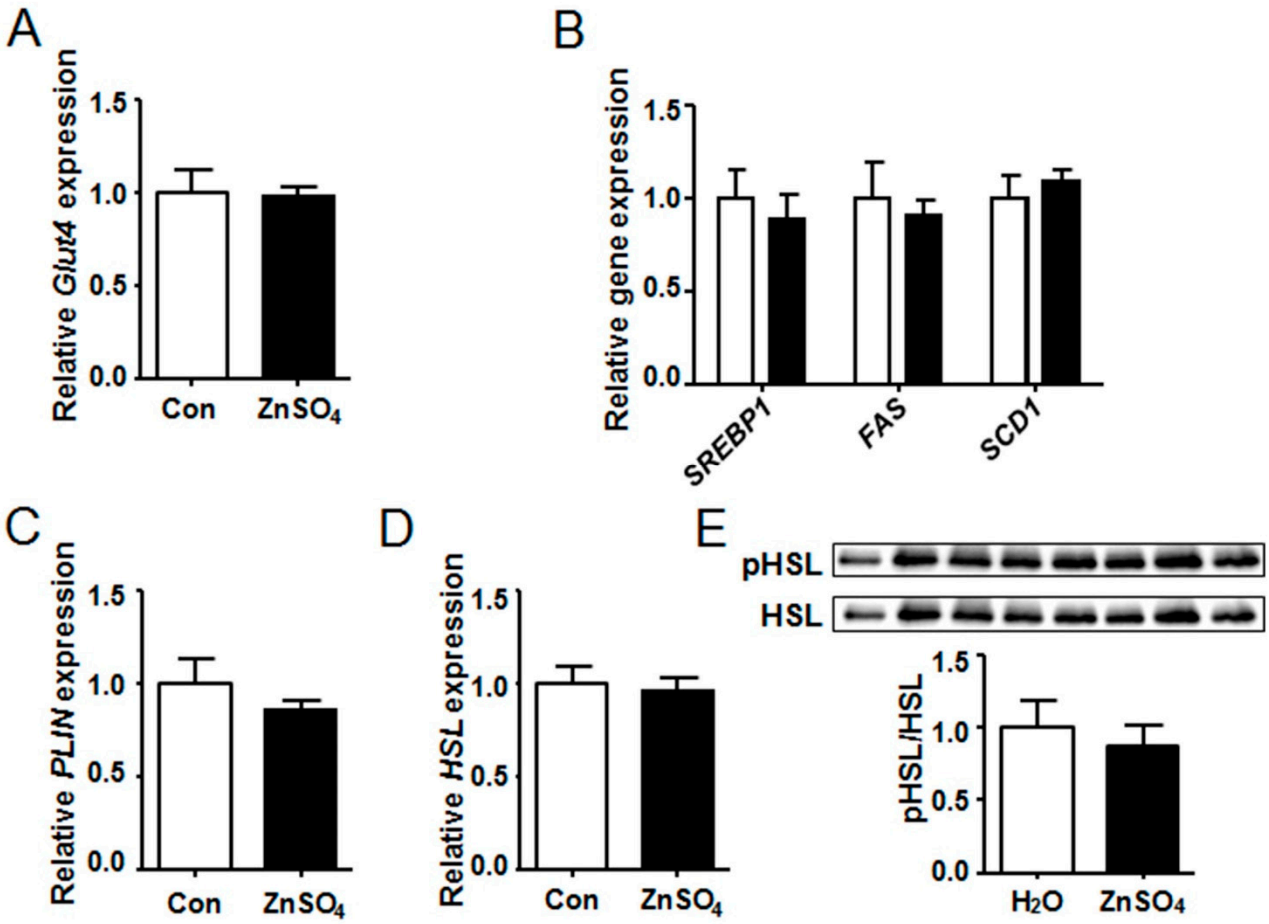

Figure S6. The expression of lipids metabolic genes in epididymal adipose tissue. (**A**) The expression of *Glut4*; (**B**) The expression of lipogenic genes; (**C**) The expression of *PLIN*; (**D**) The expression of *HSL*. (**E**) The phosphorylation levels of HSL in epdidymal adipose tissue. N=8 for each group. Data were shown as mean ± SEM. Con, control group; $ZnSO_4$, zinc sulfate-supplemented group.

Table S1 Concentration of zinc in the drinking water

Samples	Concentration of Zinc[@]	*P* value
Spring water	0.002±0.0001 (mg/L)	<0.0001
$ZnSO_4$ supplemented spring water	29.651±0.892 (mg/L)	
Feed	38.467±2.437 (mg/kg)	—

@, samples from random three times were processed to analyze the concentration of zinc.

Table S2 Water consumption (ml/cage/week)

Age (weeks)	Control (mean±SEM)	Zinc (mean±SEM)	*P* value			
			post-hoc	Zinc	Age	Zinc X Age
5	147±16	162±14	0.5344	0.1960	0.0004	0.0290
7	274±33	213±20	0.1862			
9	177±35	322±20*	0.0226			
11	143±6	228±70	0.2920			
13	198±9	179±34	0.6153			
15	200±8	213±9	0.3235			
17	212±36	278±33	0.2468			
19	315±36	280±32	0.5039			

N=3 for each group, * *P*<0.05 Zinc vs Control.

Table S3 Food intake (g/cage/week)

Age (weeks)	Control (mean±SEM)	Zinc (mean±SEM)	*P* value			
			post-hoc	Zinc	Age	Zinc X Age
5	86.50±2.58	85.70±0.81	0.7802	0.5815	<0.0001	0.0918
7	70.82±2.10	70.71±0.42	0.9615			
9	82.49±1.46	81.57±2.39	0.7592			
11	111.14±1.98	112.52±0.45	0.5340			
13	84.36±2.17	84.83±1.90	0.8785			
15	119.38±2.72	113.04±2.18	0.1431			
17	82.41±1.66	85.44±1.97	0.3058			
19	91.48±0.94	86.74±1.72	0.0724			

N=3 for each group.

Table S4 Serum zinc and copper levels

	Control (mean±SEM)	Zinc (mean±SEM)	*P* value
Serum Zinc levels (mg/L)	0.85±0.09	1.29±0.07*	0.0017
Serum Copper levels (mg/L)	0.85±0.05	0.99±0.11	0.2927
Serum Zinc/Copper ratio	1.02±0.12	1.39±0.12*	0.0462

N=8 for each group, * *P*<0.05 Zinc vs Control.

Table S5 Blood glucose levels

	Control (mean±SEM)	Zinc (mean±SEM)	*P* value
Blood glucose at fed (mMol/L)	10.34±0.80	11.40±0.86	0.3775
Blood glucose at fasting (mMol/L)	3.77±0.13	3.93±0.14	0.4223

N=8 for each group.

Table S6 Serum cytokine levels

	Control (mean±SEM)	Zinc (mean±SEM)	*P* value
Leptin (ng/ml)	1.91±0.48	3.48±0.51*	0.0420
IL6 (pg/ml)	18.17±3.56	14.56±2.81	0.3812
MCP1 (pg/ml)	54.87±19.17	34.20±8.04	0.4493
Insulin (ng/ml)	0.29±0.05	0.33±0.07	0.6040

N=8 for each group, * *P*<0.05 Zinc vs Control.

Table S7 Blood glucose levels during ITT (mMol/L)

	Control	Zinc	*P*-value			
Minutes	(mean±SEM)	(mean±SEM)	post-hoc	Zinc	Time	Zinc X Time
0	7.11±0.66	9.06±0.25*	0.0174			
15	5.53±0.38	6.07±0.30	0.2847			
30	5.20±0.49	5.09±0.20	0.8320	0.1007	<0.0001	0.0012
45	4.49±0.26	5.36±0.28*	0.0429			
60	4.66±0.28	5.24±0.17	0.1013			
90	5.24±0.19	5.27±0.25	0.9293			

N=7 for each group, * *P*<0.05 Zinc vs Control.

Table S8 Blood glucose levels during GTT (mMol/L)

	Control	Zinc	*P*-value			
Minutes	(mean±SEM)	(mean±SEM)	post-hoc	Zinc	Time	Zinc X Time
0	3.67±0.14	3.94±0.15	0.2133			
15	13.20±0.62	15.37±1.06	0.1025			
30	17.21±1.99	23.87±1.76*	0.0278	0.0318	<0.0001	0.0621
45	19.56±2.08	22.99±1.65	0.2214			
60	16.89±1.36	17.50±1.01	0.7224			
90	12.19±0.53	15.63±1.06*	0.0131			

N=7 for each group, * *P*<0.05 Zinc vs Control.

Table S9 Body weight during experiment (g)

Age (weeks)	Control (mean±SEM)	Zinc (mean±SEM)	*P* valuve			
			post-hoc	Zinc	Age	Zinc X Age
4	17.36±0.40	17.37±0.51	0.9857			
6	21.53±0.39	21.37±0.33	0.7641			
8	22.89±0.42	22.94±0.26	0.9233			
10	24.61±0.40	24.52±0.28	0.8639			
12	26.07±0.48	26.09±0.36	0.9761	0.9665	<0.0001	0.9023
14	27.16±0.59	27.14±0.34	0.9767			
16	28.22±0.71	27.84±0.29	0.6219			
18	28.92±0.66	29.51±0.36	0.4406			
20	30.16±0.66	30.34±0.54	0.8383			

N=12 for each group.

Table S10 Body weight and tissue weight at harvesting

	Control (mean±SEM)	Zinc (mean±SEM)	*P* valuve
Body weight (g)	26.34±0.43	27.17±0.40	0.1814
Liver weight (g)	1.13±0.02	1.13±0.02	0.9327
Perirenal fat weight (g)	0.15±0.02	0.24±0.02*	0.0143
Epididymal fat weight (g)	0.48±0.05	0.60±0.05	0.0767
Subcutaneous fat weight (g)	0.17±0.02	0.20±0.02	0.3032

N=8 for each group, * *P*<0.05 Zinc vs Control

Table S11 Adipocyte size (μm^2)

	Control (mean±SEM)	Zinc (mean±SEM)	*P* value
Perirenal adipocytes	2410±83	3898±102*	<0.0001
Epididymal adipocytes	2184±122	2812±149*	0.0012

N=160 for each group, * *P*<0.05 Zinc vs Control.

Table S12 Relative gene expression in the perirenal adipose tissue

Gene	Control (mean±SEM)	Zinc (mean±SEM)	*P* value
Leptin	1.00±0.26	1.95±0.26*	0.0216
IL6	1.00±0.18	1.62±0.19*	0.0297
CD11c	1.00±0.18	0.87±0.11	0.5449
F4/80	1.00±0.16	1.09±0.07	0.6018
MCP1	1.00±0.10	1.28±0.17	0.4197
Glut4	1.00±0.16	0.91±0.08	0.6276
PPARγ	1.00±0.12	0.78±0.07	0.1159
C/EBPα	1.00±0.16	0.86±0.08	0.2519
SREBP1	1.00±0.12	0.83±0.08	0.2513
FAS	1.00±0.17	0.88±0.11	0.5598
SCD1	1.00±0.21	0.61±0.07	0.0831
ACC1	1.00±0.19	0.84±0.13	0.4832
PLIN	1.00±0.05	0.94±0.04	0.3299
HSL	1.00±0.04	0.95±0.04	0.4322

N=8 for each group, * *P*<0.05 Zinc vs Control.

Table S13 Relative gene expression in the epididymal adipose tissue

Gene	Control (mean±SEM)	Zinc (mean±SEM)	*P* value
Leptin	1.00±0.14	1.64±0.23*	0.0328
IL6	1.00±0.12	1.36±0.11*	0.0482
CD11c	1.00±0.25	1.23±0.28	0.5445
F4/80	1.00±0.17	1.38±0.16	0.1338
MCP1	1.00±0.13	0.95±0.07	0.7337
Glut4	1.00±0.12	0.98±0.05	0.8662
SREBP1	1.00±0.16	0.89±0.13	0.5898
FAS	1.00±0.19	0.91±0.08	0.6741
SCD1	1.00±0.12	1.09±0.06	0.5346
PLIN	1.00±0.13	0.86±0.05	0.3212
HSL	1.00±0.09	0.96±0.07	0.7488

N=8 for each group, * *P*<0.05 Zinc vs Control.

Table S14 Relative gene expression in 3T3-L1 adipocytes treated with zinc sulfate

	Control (mean±SEM)	$ZnSO_4$ (mean±SEM)			*P* value			
					post-hoc			one-way
Gene		10 μM	50 μM	100 μM	10 μM	50 μM	100 μM	ANOVA
Leptin	1.00±0.08	0.78±0.20	1.52±0.13*	1.78±0.36	0.3567	0.0238	0.0752	0.0271
IL6	1.00±0.07	1.51±0.13*	3.48±0.30*	2.84±0.09*	0.0250	0.0013	0.0001	<0.0001

N=3 for each treatment, * *P*<0.05 Zinc vs Control.

Table S15 Relative gene expression in 3T3-L1 adipocytes treated with zinc chloride

	Control (mean±SEM)	$ZnCl_2$ (mean±SEM)	*P* value
Leptin	1.00±0.08	1.62±0.21*	0.0195
IL6	1.00±0.13	1.71±0.13*	0.0030

N=3 for each treatment, * *P*<0.05 Zinc vs Control.

Table S16 Relative protein levels and protein phosphorylation levels in perirenal adipose tissue

	Control (mean±SEM)	Zinc (mean±SEM)	*P* value
pAKT T308/AKT	1.00±0.23	0.21±0.04*	0.0040
pAKT S473/AKT	1.00±0.19	1.02±0.27	0.9445
PPARγ/Tubulin	1.00±0.31	1.02±0.40	0.5472
FAS/Tubulin	1.00±0.25	0.22±0.06*	0.0006
SCD1/Tubulin	1.00±0.40	0.23±0.12	0.0723
pHSL/HSL	1.00±0.14	1.37±0.11	0.0560

N=8 for each group, * *P*<0.05 Zinc vs Control.

Table S17 Relative protein levels and protein phosphorylation levels in epididymal adipose tissue

	Control (mean±SEM)	Zinc (mean±SEM)	*P* value
pAKT T308/AKT	1.00±0.29	1.45±0.37	0.2618
pAKT S473/AKT	1.00±0.26	0.93±0.36	0.6893
pHSL/HSL	1.00±0.18	0.87±0.15	0.5744

N=8 for each group, * *P*<0.05 Zinc vs Control.

Table S18 Relative phosphorylation levels of AKT in 3T3-L1 adipocytes

	Veh		Insulin		Tow-way ANOVA			post-hoc *P* value			
								$ZnSO_4$ vs Con		Insulin vs Veh	
	Con	ZnSO4	Con	$ZnSO_4$	$ZnSO_4$	Insulin	Interaction	Veh	Insulin	Con	$ZnSO_4$
pAKT T308/AKT	0.00±0.00	5.10±1.03*	1.00±0.25	30.66±3.29*	< 0.0001	< 0.0001	0.0001	0.0077	0.0008	0.0164	0.0018
pAKT S473/AKT	0.00±0.00	1.98±0.48*	1.00±0.12	34.60±2.09*	< 0.0001	< 0.0001	< 0.0001	0.0150	0.0001	0.0012	0.0001

N=3 for each treatment, * *P*<0.05 Zinc vs Control.

Table S19 Serum TAG and FFA levels

	Control (mean±SEM)	Zinc (mean±SEM)	*P* value
TAG (mMol/L)	1.66±0.06	1.51±0.08	0.1516
FFA (mMol/L)	1.25±0.05	1.17±0.07	0.3726

N=8 for each group.