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Maternal plasma L-carnitine reduction during pregnancy is mainly attributed to OCTN2 mediated placental uptake and does not result in maternal hepatic fatty acid β-oxidation decline

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Running title: The mechanism of maternal plasma L-carnitine reduction

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Abbreviations: Acsl1, long-chain acyl-CoA synthetase 1; $ATB^{0,+}$, amino acid transporter $B^{0,+}$; Bdh1, 3-hydroxybutyrate dehydrogenase 1; CoA, coenzyme A; CORT, corticosterone; Cpt1a, carnitine palmitoyltransferase 1a; CT1, carnitine transporter 1; DMEM, Dulbecco's Modified Eagle's Medium; E2, estradiol; FBS, fetal bovine serum; GD, gestation day; Hmgcl, hydroxymethyl glutaryl-CoA lyase; Hmgcs2, hydroxymethyl glutaryl-CoA synthase; L-Car, L-carnitine; d_3-L-Car, d_3-L-carnitine; MDCK, Madin–Darby canine kidney; LD, lactation day; OCTN2,

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carnitine/organic cation transporter 2; P4, progesterone; PPARα, peroxisome proliferator-activated receptor α; qRT-PCR, quantitative real-time polymerase chain reaction; SDS, sodium dodecyl sulfate; TG, triglyceride; TC, total cholesterol.

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ABSTRACT

L-Carnitine (L-Car) plays a crucial role in fatty acid β-oxidation. However, the plasma L-Car concentration in women markedly declines during pregnancy, the underlying mechanism and the consequent on maternal hepatic β-oxidation has not been clarified yet. Our results showed that the plasma L-Car level in mice at gestation day (GD) 18 was significantly lower than that in non-pregnant mice, and the mean fetal-to-maternal plasma L-Car ratio in GD 18 mice was 3.0. Carnitine/organic cation transporter 2 (OCTN2) was highly expressed in mouse and human placenta and up-regulated as gestation proceeds in human placenta, while carnitine transporter (CT) 1, CT2 and amino acid transporter $B^{0,+}$ ($ATB^{0,+}$) were extremely low. Further study revealed that renal peroxisome proliferator-activated receptor α (PPARα) and OCTN2 were down-regulated, renal L-Car level was reduced while the urinary excretion of L-Car was lower in late pregnant mice than that in non-pregnant mice. Meanwhile, progesterone (pregnancy-related hormone) down-regulated the expression of renal OCTN2 *via* PPARα-mediated pathway, and inhibited the activity of OCTN2, but estradiol, corticosterone and cortisol did not. Unexpectedly, the maternal hepatic level of L-Car, β-hydroxybutyrate (an indicator of mitochondrial β-oxidation) and mRNA level of several enzymes involved in fatty acid β-oxidation in GD 18 mice were higher than that in non-pregnant mice. In conclusion, OCTN2 mediated L-Car transfer across placenta played a major role in maternal plasma L-Car reduction during pregnancy, which did not subsequently result in maternal hepatic fatty acid β-oxidation decrease.

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Introduction

L-Carnitine (L-Car) is a hydrophilic quaternary amine that plays a crucial role in fatty acid β-oxidation. L-Car and long-chain acyl coenzyme A (CoA) form acylcarnitines by the action of carnitine palmitoyltransferase 1. Acylcarnitines are then translocated across the mitochondrial membrane by the carnitine/acylcarnitine translocase. Once inside mitochondria, carnitine palmitoyltransferase 2 removes L-Car from acylcarnitines and re-generates acyl-CoAs. L-Car then returns to the cytoplasm for another cycle while the acyl-CoAs enter β-oxidation (Ramsay et al., 2001; Foster, 2004). L-Car deficiency may cause fatty acid oxidation disorder, and lead to cardiomyopathy, muscle weakness, hypoglycemia and fatty liver (Frigeni et al., 2017). The plasma concentration of L-Car in pregnant women is reported to be much lower than that in non-pregnant women and keeps decline as gestation proceeds (Winter et al., 1995; Lindsay et al., 2015). At the time of delivery, the maternal plasma L-Car concentration is only half of that in non-pregnant women, which will be returned to its original level 8 weeks after delivery (Cederblad et al., 1986; Cho and Cha, 2005). It seems that pregnancy leads to a reversible secondary deficiency of L-Car (Marzo et al., 1994). However, the reasons have not been clarified yet.

In humans, L-Car homoeostasis is maintained by dietary absorption, endogenous synthesis, and efficient renal reabsorption (Vaz and Wanders, 2002). About 75% of L-Car requirement is diet provided, while 25% is synthesized endogenously in the liver and kidney of adults. L-Car is eliminated in its original form by glomerular filtration in human, however, most of the filtered L-Car will be reabsorbed in kidney

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by carnitine transporters, especially by carnitine/organic cation transporter 2 (OCTN2, SLC22A5), a high affinity L-Car transporter located in the apical side of renal tubular epithelial cells (Longo et al., 2016). A defect in the OCTN2 will cause urinary L-Car wasting and subsequently result in primary L-Car deficiency (Lahjouji et al., 2002). Besides OCTN2, carnitine transporter 1 (CT1, SLC22A15), CT2 (SLC22A16), and amino acid transporter $B^{0,+}$ ($ATB^{0,+}$, SLC6A14) can also mediate L-Car uptake (Sekine et al., 1998; Nakanishi et al., 2001; Enomoto et al., 2002). Therefore, carnitine transporters in the kidney are very important for the L-Car homeostasis.

A series of physiologic changes, including increased maternal fat, blood volume, cardiac output, and blood flow to the kidneys, occur in pregnant women (Costantine, 2014). In addition, the plasma concentrations of several hormones are gradually increased during pregnancy. The plasma levels of estradiol (E2) and progesterone (P4) in pregnant women during the third trimester will reach tens to hundreds folds of the original levels (La Marca et al., 2005; Zhang et al., 2015). And the plasma concentrations of glucocorticoids (cortisol in humans and corticosterone (CORT) in rodents) are also gradually increased during pregnancy (Soldin et al., 2005; Jung et al., 2011).

It is noteworthy that the placenta mediates maternofetal transport of a variety of nutrients, including L-Car. L-Car is pivotal for the fetal organism, but fetus has limited capacity for L-Car biosynthesis (Hahn, 1981; Shenai and Borum, 1984), thus, fetus obtains almost all of the required L-Car from the maternal circulation. And the L-Car level in umbilical cord plasma is reported to be higher than that in the mother

(Talian et al., 2007; Keller et al., 2009). We hypothesized that it might be one of the reasons for the reduction of maternal plasma L-Car concentration during pregnancy. It has been reported that OCTN2 which located in the apical membrane of syncytiotrophoblasts of human placenta mediates the placental transport of L-Car (Lahjouji et al., 2004; Grube et al., 2005), however, it is not clear whether other carnitine transporters are involved in the process.

It was known that plasma lipid levels, including triglyceride (TG) and total cholesterol (TC), were markedly higher in pregnant women than that in non-pregnant women, and were increased as gestation proceeds (Alemu et al., 2018; Wang et al., 2018). In comparison with healthy controls, the levels of TG and TC were much higher in women with preeclampsia, gestational diabetes mellitus and intrahepatic cholestasis of pregnancy (Pecks et al., 2016; Layton et al., 2019). However, the mechanism also remains unknown.

Based on the above information, we hypothesized that L-Car deficiency during pregnancy might induce hepatic fatty acid oxidation disorder, and subsequently lead to the increase of plasma lipid levels. Therefore, one aim of the present study was to elucidate a possible mechanism for the reduction of maternal plasma L-Car concentration during gestation, the other aim was to explore whether such low L-Car concentration associated with plasma lipid levels rise in pregnancy.

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Materials and methods

Materials

Fetal bovine serum (FBS), trypsin, Dulbecco's Modified Eagle's Medium (DMEM) and DMEM/F12 were purchased from GIBCO Invitrogen Life Technologies (Grand Island, NY, USA). L-carnitine (L-Car) was purchased from Meilun Biological Co. Ltd. (Dalian, China). L-carnitine (methyl-d_3) (d_3-L-Car) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). E2, P4, cortisol and WY14643 were purchased from Aladdin biochemical technology co., LTD (Shanghai, China). Corticosterone (CORT) was obtained from TCI (Shanghai) Development Co., Ltd. OCTN2 antibody (species reactivity: human, mouse, rat) was obtained from Sigma-Aldrich (St. Louis, MO, USA. (SAB4300885)). GAPDH antibody and the secondary anti-rabbit and anti-mouse antibodies were obtained from Multi Sciences Biotech Corporation (Hangzhou, China). Acetonitrile was obtained from Tedia (Fairfield, OH, USA). Ammonium acetate and formic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals or solvents were of the highest grade commercially available.

Animal experiments

All animal procedures were performed in accordance with the 'Principles of laboratory animal care' (http://grants1.nih.gov/grants/olaw/references/phspol.htm) and were approved by the Institutional Animal Care and Use Committee of Zhejiang University (2015-0026). All experimental procedures were conducted as humanely as possible.

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Specific pathogen free female non-pregnant and pregnant (gestational day 14, GD 14) ICR mice, age of 8 – 10 weeks, were obtained from Beijing Vital River Laboratory Animal Technology Company (Beijing, China) [SCXK (Jing) 2016-0011]. The animals were housed in cages at controlled temperature (22.0 ± 1°C) and humidity (50 ± 10) % with a 12-h light-dark cycle and free access to food and water throughout the study. Mice consumed ad libitum a commercial standard mouse diet (Laboratory Rodent Diet 5001; LabDiet). No dietary manipulations were otherwise conducted and all dietary conditions were the same for the control and treated groups.

Blood, livers, kidneys, hearts and muscles of the non-pregnant (n = 10) and pregnant (GD 18, n = 10) mice were collected after cervical dislocation, and the fetal blood samples were collected immediately after fetuses were sacrificed by decapitation. The plasma/serum was collected immediately after centrifugation at 8000 g for 10 min and stored at -40 ℃ until analysis. Placentas of the pregnant mice at GD 15 (n = 8) and 18 (n = 8) were collected and the tissues were frozen in liquid nitrogen immediately after dissection and stored at -80 ℃ until analysis.

Another non-pregnant mice (n = 10, 2 mice per cage) and pregnant mice (n = 5, 1 mouse per cage) were used in the urine excretion study. On GD 16 – 18 and lactation day (LD) 8 – 10, urine samples within 24 h from pregnant or postpartum mice were collected. On another successive 3 days (day1 – day3), urine samples within 24 h from non-pregnant mice were collected.

The concentrations of L-Car in plasma, tissues and urine were determined by the LC-MS/MS method described previously (Wang et al., 2019). The mRNA and protein

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levels of related genes in the liver, kidney and placenta were measured by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot. The concentrations of β-hydroxybutyrate in serum and liver were determined using a β-Hydroxybutyrate Test Kit（Colorimetric Method）(Ningbo Ruiyuan Biotechnology co., LTD, Ningbo, China).

Cell culture

Parental Madin–Darby canine kidney (MDCK) cells were obtained from Peking Union Medical College (Beijing, China). MDCK-hOCTN2 cells (MDCK cells stably transfected with plasmid pcDNA3.1 (+) vector containing human OCTN2 cDNA) were constructed in our laboratory (Li et al., 2017). Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37℃ with 5% (v/v) CO_2. HK-2 cells were obtained from Stem Cell Bank and maintained in DMEM/F12 medium with 10% FBS and 1% penicillin/streptomycin in a humidified 5% (v/v) CO_2 air atmosphere at 37℃.

Cellular accumulation experiment

MDCK-hOCTN2 cells were seeded into 24-well plates at a density of 2×10^5/well. On day three after seeding, cellular accumulation experiments were performed with the method described by our laboratory (Bai et al., 2017; Ma et al., 2017). Cells were washed twice and pre-incubated with Krebs-Ringer-Henseleit buffer (KRH) for 10 min at 37°C, then 200 μL KRH containing d_3-L-Car with or without pregnancy-related hormones (E2, P4, CORT and cortisol) was added to initiate accumulation for 3 min. Accumulation was terminated by removing the incubation

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buffer and immediately adding ice-cold buffer. Finally, cells were washed three times with ice-cold buffer and lysed with 100 μL 0.1% sodium dodecyl sulfate (SDS). The accumulation assay in HK-2 cells was similar to that in MDCK-hOCTN2 cells. The concentration of d_3-L-Car was measured using LC-MS/MS with the method described previously (Wang et al., 2019).

Treatment of HK-2 cells with hormones

HK-2 cells were seeded at a density of 4×10^5/well in 12-well plates. After overnight, cells were treated with or without designated concentrations of E2, P4, CORT or cortisol for 24h (refreshing the medium per 12 h), then the concentration of L-Car or mRNA expression of OCTN2 were measured. To investigate the role of peroxisome proliferator-activated receptor α (PPARα) in OCTN2 expression, the cells were incubated with 100 μM WY14643 (a PPARα agonist) under 20μM P4 treatment.

Clinical Tissue Collection

All the human placentas were obtained from uncomplicated pregnancies in accordance with ethical standards at Women's Hospital, School of Medicine, Zhejiang University. All the participants provided written informed consent. Term placentas (n = 23) were collected following natural delivery at term (38 – 40 week of gestation) and the first trimester placentas (n = 32) were acquired from interruption of the pregnancy between 8 and 10 week of gestation. For qRT-PCR and western blot, the placental samples were frozen in liquid nitrogen immediately after delivery or surgery and stored at –80 ℃ until analysis. For immunohistochemistry, placental tissue sections were obtained by dissecting a segment (1.5 cm square, 0.5 cm thick) from the

maternal part of the placenta and were fixed in 4% formaldehyde at 4 ℃ for 2 days, and paraffin sections of 2 μm were generated.

qRT-PCR assay

Total RNA was extracted from tissues and cells using RNA simple Total RNA Kit (Tiangen, China). Then cDNA was synthesized according to the manufacturer's protocol using PrimeScript RT Master Mix (TaKaRa, Tokyo, Japan). The resulting cDNA was amplified by qRT-PCR with SYBR® Premix EX Taq (Takara, Tokyo, Japan). Relative mRNA levels of target genes were normalized by GAPDH, using the ΔCt method and described as $2^{-\Delta Ct}$, ΔCt = Avg. Ct (Target gene) – Avg. Ct (GAPDH). To determine the fold-change of target genes, results were calculated using the ΔΔCt method and described as $2^{-\Delta\Delta Ct}$, ΔΔCt= (Avg. Ct (Target gene) – Avg. Ct (GAPDH))$_{sample}$ – (Avg. Ct (Target gene) – Avg. Ct (GAPDH))$_{control}$. The specific primers were showed in Supplemental Table 1.

Western blot analysis

Tissues were homogenized in RIPA lysis buffer (Beyotime, Shanghai, China), and the protein concentration was determined with a BCA Protein Assay Kit (Beyotime, Shanghai, China). Proteins were mixed with loading buffer (P0015; Beyotime, Nanjing, China) and then boiled at 100 ℃ for 5 min. Aliquots of denatured protein were separated by SDS-PAGE (Bio-Rad, Hercules, CA) and subsequently transferred onto PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membranes were blocked and incubated with primary anti-OCTN2 antibody (1:1000) or anti-GAPDH antibody (1:5000) for 4 h at room temperature. Membranes were then

washed with TBST 3 times (5 min every time) and incubated with HRP-conjugated secondary antibodies at room temperature for 2 h. After being washed with TBST again 3 times (5 min every time), signals were visualized by chemiluminescent detection using the enhanced chemiluminescence (ECL) Western blotting detection system (LI-COR Biosciences, Lincoln, NE).

Immunohistochemistry

The slides were deparaffinized in xylene and rehydrated in a series of graded alcohols. After quenching endogenous peroxidase activity with 1% H_2O_2, the sections were rinsed with PBS and incubated with 5% BSA for 30 min at room temperature to reduce nonspecific binding, then for 16 h at 4 °C with anti-OCTN2 antibody (1:50) diluted in 5% BSA. The section was incubated with biotinylated anti-rabbit IgG antibody for 1 h at room temperature, washed, then incubated with streptavidin peroxidase for 1 h at room temperature, and followed by interaction with the DAB (3,3-diaminobenzidine) liquid substrate system (Sigma, MO, USA) for 1 min. All sections were counterstained with hematoxylin for 3 min.

Data analysis

Data are expressed as mean ± SEM. *In vitro* experiments were conducted at least two times in triplicate. IC_{50} values were assessed by nonlinear regression using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Student's *t*-test was used to compare differences between two groups, while one-way ANOVA with the Dunnett's test was applied for data with more than two groups. *P* values < 0.05 were considered statistically significant.

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Results

The maternal plasma L-Car concentration decreased in pregnant mice

In non-pregnant mice, plasma L-Car concentration was about 19.5 μM, however, it was sharply reduced to 12.6 μM in pregnant mice at gestation day (GD) 18 ($P < 0.01$, Fig. 1).

Reduction of maternal plasma L-Car level might be attributed to high placental transfer *via* OCTN2

To clarify the reason for maternal plasma L-Car reduction in pregnant mice, we measured the concentrations of L-Car in the maternal and fetal plasma. As shown in Fig. 2A, the mean concentrations of L-Car were 12.6 μM in maternal plasma and 37.8 μM in fetal plasma, respectively. The mean fetal-to-maternal plasma concentration ratio of L-Car in GD 18 mice was 3.0. Since placental transfer of L-Car is mainly mediated by carnitine transporters, we detected the mRNA levels of Ct1, Ct2, Octn2 and $Atb^{0,+}$ in the placenta of pregnant mice at GD 15 and GD 18 (Fig. 2B). Our results revealed that Octn2 was highly expressed in mouse placenta, but no difference was found between GD 15 and GD 18 mice. The mRNA level of Ct1 and $Atb^{0,+}$ were extremely low in mouse placenta, while Ct2 was undetectable. The above data implies that OCTN2 plays a key role in placental transfer of L-Car.

We further detected the mRNA expression of carnitine transporters in human placenta. As shown in Fig. 2C, OCTN2 was highly expressed, while CT1 and CT2 were very low and $ATB^{0,+}$ was undetected. The mRNA and protein expression of OCTN2 in the term placenta were obviously higher than that in the first trimester

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placenta (Fig. 2D). The immunohistochemical results showed that OCTN2 was expressed on the maternal side of syncytiotrophoblasts of the human term placenta (Fig. 2E). Therefore, OCTN2 also plays a crucial role in L-Car transfer across human placenta.

Reduction of maternal plasma L-Car level might not be attributed to renal excretion increase

L-Car is excreted into urine by glomerular filtration, however, most of the filtered L-Car is reabsorbed in proximal tubules *via* transporters. Thus, a decrease in renal reabsorption of L-Car will also lead to plasma L-Car reduction. However, the urinary excretion of L-Car in pregnant mice during the third trimester (GD 16 – GD 18) was tremendously lower than that in non-pregnant mice (day 1 – day 3), while the excretion largely increased after delivery (LD 8 – LD 10) (Fig. 3A). The results indicate that the decreased concentration of L-Car in maternal plasma of pregnant mice in the third trimester is not attributed to urinary excretion increase.

The concentration of L-Car in the kidney of GD 18 mice (326 nmol/g) was significantly lower than that in non-pregnant mice (551 nmol/g) ($P < 0.001$, Fig. 3B). Moreover, the mRNA level of Octn2 in the kidney of GD 18 mice was approximately 72% of that in non-pregnant mice ($P < 0.001$), whereas the mRNA expressions of other carnitine transporters including Ct1, Ct2 and $Atb^{0,+}$ were very low in the kidney (Fig. 3C). The results suggest that renal reabsorption of L-Car is decreased in late pregnancy due to down-regulated OCTN2 in the kidney.

OCTN2 expression was down-regulated by P4 in HK-2 cells

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To investigate the reason why renal Octn2 was down-regulated in GD 18 mice, we further explored whether pregnancy-related hormones including E2, P4, CORT and cortisol had effect on OCTN2 expression in HK-2 (a normal human proximal tubule epithelial cell line) cells. P4 (5 – 20 μM) concentration-dependently down-regulated the mRNA level of OCTN2, while E2 did not show any effect (Fig. 4, A and B). However, 10 μM CORT and cortisol up-regulated the mRNA level of OCTN2 in HK-2 cells (Fig. 4C). In addition, P4 reduced the concentration of L-Car in HK-2 cells with the IC_{50} value of 10.7 μM (Fig. 4D).

P4-induced down-regulation of OCTN2 was probably through down-regulation of PPARα but not PPARγ

It has been reported that PPARα and PPARγ contributed to OCTN2 regulation (D'Argenio et al., 2010; Luo et al., 2014; Luo et al., 2016). In order to determine whether PPARα/γ contributed to P4-induced OCTN2 down-regulation, we examined the mRNA expression of PPARα/γ in HK-2 cells with or without P4 treatment. P4 (5 – 20 μM) down-regulated the mRNA level of PPARα in a concentration dependent manner, whereas the PPARγ expression was not markedly altered (Fig. 5, A and B). Furthermore, WY14643 (100 μM), a PPARα agonist, blocked 20 μM P4-induced down-regulation of OCTN2 as well as PPARα in HK-2 cells ($P < 0.001$, Fig. 5, C and D). Additionally, the mRNA level of Pparα was also obviously lower in the kidney of GD 18 mice than that in non-pregnant mice ($P < 0.05$, Fig. 5E). The above results suggest that P4 down-regulates the expression of OCTN2 in HK-2 cells through PPARα-mediated pathway.

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P4 inhibited OCTN2 mediated d_3-L-Car uptake

To determine whether pregnancy-related hormones inhibit the L-Car uptake mediated by OCTN2, the accumulation of d_3-L-Car in MDCK-hOCTN2 cells were evaluated in the absence or presence of E2, P4, CORT or cortisol. Our data demonstrated that P4 reduced the accumulation of d_3-L-Car in a concentration-dependent manner with the IC_{50} value of 15.8 μM, whereas E2 did not (Fig. 6, A and B). High concentration (100 μM) of CORT and cortisol slightly inhibited the uptake of d_3-L-Car in MDCK-hOCTN2 cells ($P < 0.001$, Fig. 6C). Similarly, P4 (10 and 50 μM) significantly reduced the uptake of d_3-L-Car in HK-2 cells ($P < 0.001$, Fig. 6D).

β-Oxidation of fatty acids in the liver of GD 18 mice was higher than that in non-pregnant mice

Although the concentration of L-Car in maternal plasma was much lower in GD 18 mice than that in non-pregnant mice (Fig. 1), the L-Car concentration in the liver of GD 18 mice was significantly higher than that in non-pregnant mice ($P < 0.01$, Fig. 7A). Similarly, the hepatic level of β-hydroxybutyrate, an indicator of mitochondrial β-oxidation, was also obviously higher in GD 18 mice than that in non-pregnant mice ($P < 0.001$, Fig. 7B), while the serum level was not markedly altered ($P > 0.05$, Fig. 7C). To further elucidate the mechanism of hepatic β-hydroxybutyrate increase, the mRNA levels of key enzymes involved in fatty acid β-oxidation were measured. As shown in Fig. 7D, the long-chain acyl-CoA synthetase 1 (Acsl1) and carnitine palmitoyltransferase 1a (Cpt1a) in the liver were up-regulated obviously in GD 18

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mice compared to non-pregnant mice ($P < 0.01$), however, the expressions of other genes were not changed ($P > 0.05$). In addition, the enzymes involved in β-hydroxybutyrate synthesis, such as hydroxymethyl glutaryl-CoA synthase (Hmgcs2) and hydroxymethyl glutaryl-CoA lyase (Hmgcl), were significantly increased in the liver of GD 18 mice ($P < 0.01$), while 3-hydroxybutyrate dehydrogenase 1 (Bdh1) decreased ($P < 0.05$) (Fig. 7E). These results suggest that β-oxidation of fatty acids in the liver of GD 18 mice is higher than that in non-pregnant mice.

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Discussion

The present study investigated the mechanism of maternal plasma L-Car concentration reduction during pregnancy in mice, and found that OCTN2 mediated L-Car transfer across placenta played a major role in maternal plasma L-Car reduction, and the reduction of maternal plasma L-Car level did not induce decrease in hepatic L-Car concentration and fatty acid β-oxidation.

Our study found that the plasma concentration of L-Car in GD 18 mice was only 65% of that in non-pregnant mice (Fig. 1), which was consistent with the results in pregnant and non-pregnant women (Cederblad et al., 1986; Cho and Cha, 2005). We subsequently found that L-Car concentration in fetal plasma was 3 folds of that in maternal plasma of GD 18 mice (Fig. 2A). Although CT1, CT2, OCTN2 and $ATB^{0,+}$ can mediate L-Car uptake, however, our results revealed that only Octn2/OCTN2 mRNA was highly expressed in mouse and human placenta (Fig. 2, B and C), which indicated that OCTN2 might mediate most of the maternal–fetal L-Car transport. And the expression of OCTN2 in human placenta was up-regulated as gestation proceeds (Fig. 2D), which is possibly due to the increased demand of fetus for L-Car. In addition, although OCTN1 (SLC22A4) is expressed in the placenta and has high homology to OCTN2 (Tamai et al., 1997), however, several published studies showed that human/mouse/rat OCTN1 did not (Wu et al., 2000; Amat et al., 2003) or transported L-Car with a very low capacity (Yabuuchi et al., 1999; Tamai et al., 2000). Our study demonstrated that d_3-L-Car was not a substrate of hOCTN1 using the MDCK-hOCTN1 cell model (Supplemental Fig. 1A). Moreover, we found that L-Car

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concentration in MDCK-hOCTN1 cells was not higher than that in mock cells, while MDCK-hOCTN2 cells extremely higher (Supplemental Fig. 1B). Given the above, we considered that OCTN1 played a very limited role in L-Car placental transport. Therefore, we reasoned that OCTN2 located in the brush border membrane of placenta mediated L-Car uptake from maternal circulation, caused maternal plasma L-Car reduction. Future studies will be needed to elucidate how L-Car transports from the placenta to fetal circulation.

The increased renal excretion of L-Car can also lead to the reduction of maternal plasma L-Car. However, our following experiment revealed that the urinary excretion of L-Car in pregnant mice during the third trimester was lower than that in non-pregnant and postpartum mice (Fig. 3A). The result is in line with several previous reports (Marzo et al., 1994; Cho and Cha, 2005), in which they reported that pregnant women had higher urinary excretion of L-Car during the first trimester than that in non-pregnant or postpartum women, but the excretion was decreased markedly during the third trimester. Therefore, renal excretion of L-Car can be ruled out as possible reasons for the lower plasma L-Car concentration during late pregnancy in mice.

We found that the L-Car concentration and mRNA level of Octn2 in the kidney of GD 18 mice was significantly lower than that in non-pregnant mice (Fig. 3, B and C), which means that the renal reabsorption of L-Car in GD 18 mice was decreased. Our results demonstrated that P4 significantly inhibited the uptake of d_3-L-Car in MDCK-hOCTN2 cells and HK-2 cells (Fig. 6, B and D), which is in consistent with

the previous report that P4 was a competitive inhibitor of L-Car uptake in BeWo cells (Rytting and Audus, 2008). Furthermore, we found that P4 down-regulated the expression of OCTN2 in HK-2 cells through PPARα-mediated pathway (Fig. 5C). Meanwhile, the mRNA level of Pparα was obviously lower in the kidney of GD 18 mice than that in non-pregnant mice (Fig. 5E). Thus, the reduction of L-Car reabsorption might be the result of P4-induced down-regulation and activity inhibition of renal OCTN2. In addition, we considered that the reduction of urinary excretion of L-Car might be due to reduced glomerular filtration of L-Car because of the decreased maternal plasma L-Car concentration in late pregnancy.

Besides renal excretion and placental transfer, L-Car homeostasis in humans is also maintained by dietary intake and endogenous synthesis. Cho and Cha (2005) reported that pregnant Korean women had lower L-Car intakes than non-pregnant women during the first trimester, but increased as gestation proceeds. L-Car biosynthesis involves a complex series of reactions in several tissues. The availability of γ-butyrobetaine in the liver and kidney is limiting for L-Car biosynthesis in humans (Rebouche et al., 1989). Ringseis et al. (2010) examined plasma samples of 79 pregnant women at delivery and found a positive correlation between plasma concentration of γ-butyrobetaine and L-Car, thus they proposed that low plasma concentration of L-Car in pregnant women might be the result of a diminished L-Car biosynthesis due to the lack of sufficient γ-butyrobetaine. However, Coskun and Dogan (2002) found that L-Car biosynthesis in rabbit liver increased gradually in late pregnancy due to increased γ-butyrobetaine hydroxylase activity. Fernandez (1989)

reported that liver L-Car levels were increased during late gestation in rats. There is no report regarding the L-Car biosynthesis alteration in kidney during pregnancy. Therefore, the status of L-Car biosynthesis in human liver and kidney during pregnancy remains to be investigated. In addition, volume dilution caused by plasma volume increase during pregnancy might also contribute to maternal plasma L-Car reduction.

Based on the above consideration, we though the reasons for plasma L-Car concentration decrease in women during pregnancy are complicated. The reduction of plasma L-Car concentration in pregnant women during the first trimester might be due to dietary intake reduction and urinary excretion increase, and the reduction during the third trimester might be mainly attributed to placental transfer increase as well as volume dilution.

Because L-Car plays an important role in fatty acid β-oxidation, it is reasonable to be speculated that plasma L-Car concentration reduction during pregnancy might lead to β-oxidation decrease and plasma lipids rise. However, the hepatic level of β-hydroxybutyrate was significantly higher in GD 18 mice than that in non-pregnant mice (Fig. 7B), which means that the β-oxidation of fatty acids in the liver of GD 18 mice was higher than that of non-pregnant mice. We considered that it might be due to the increased expression of Acsl1, Cpt1a, Hmgcs2 and Hmgcl, and the higher concentration of L-Car in the liver of GD 18 mice (Fig. 7, A, D and E). On the other hand, L-Car level in the heart of GD 18 mice was lower than that of non-pregnant mice, while no difference was found in the muscle (Supplemental Fig. 2).

Nevertheless, Liu et al. (2017) found that fatty acid uptake and oxidation in the heart of late pregnant mice were increased. Taken together, we considered that decreased plasma L-Car levels might not be responsible for elevated plasma TG and TC in pregnancy. Since elevated TG levels throughout pregnancy have been shown to increase the risk of adverse pregnancy outcomes, including gestational diabetes mellitus, preeclampsia and preterm birth (Spracklen et al., 2014; Ryckman et al., 2015; Zheng et al., 2018), the mechanisms of plasma TG and TC rise during pregnancy are already under study in our lab.

It is unknown whether such low L-Car concentrations in pregnant women will induce adverse consequences. Nevertheless, it is possible that a higher L-Car status could have beneficial effects on pregnant women and their fetuses due to the important functions of L-Car. Thus, a strict vegetarian diet should be avoided during pregnancy because vegetables contain much less L-Car than meat and fish (Pochini et al., 2019). It has been reported that L-Car supplementation can prevent the marked decrease of plasma L-Car concentration and the striking increase of plasma free fatty acids in pregnant women (Keller et al., 2009). However, it remains unknown whether supplementation of L-Car will reduce plasma TG and TC in pregnant women, which needs further study.

In conclusion, our results confirmed that maternal plasma L-Car concentration was reduced in pregnancy, which was mainly attributed to placental transfer of L-Car mediated by OCTN2 but not renal excretion increase, and OCTN2 was up-regulated as gestation proceeds in human placenta. Unexpectedly, the reduction of maternal

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plasma L-Car level did not induce decrease in hepatic L-Car concentration and fatty acid β-oxidation in mice, thus it was not the main cause of plasma lipid levels rise during pregnancy.

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Authorship Contributions

Participated in research design: Bai, Sun, Zheng, S. Zeng and Jiang

Conducted experiments: Bai, Q. Zeng, Y. Chen, M. Chen and Li

Performed data analysis: Bai, Ma, Zhou and Jiang

Wrote or contributed to the writing of the manuscript: Bai, Q. Zeng and Jiang

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Footnotes

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Legends for Figures

Fig.1 The concentration of L-Car in plasma of non-pregnant and pregnant mice at GD 18. Data are expressed as mean ± SEM, n = 10. Compared with the non-pregnant group, $^{**}P < 0.01$.

Fig. 2 (A) The concentration of L-Car in maternal and fetal plasma of mice at GD 18. Data are expressed as mean ± SEM, n = 10. (B) The mRNA level of carnitine transporters in mouse placenta at GD 15 and 18. Data are expressed as mean ± SEM, n = 8. Compared with the GD 15 placenta, $^{***}P < 0.001$. (C) The mRNA level of carnitine transporters and protein level of OCTN2 (D) in the first trimester placenta and term human placenta. Data are expressed as mean ± SEM (n = 32 for first trimester placenta and n = 23 for term placenta). Compared with the first trimester placenta, $^{***}P < 0.001$. ND, not detected. (E) Immunohistochemical detection of OCTN2 in the human term placenta. Representative images shown were captured at 200 × (left panel) and 400 × (right panel) magnification.

Fig. 3 (A) L-Car excretion in mouse urine. On gestational day (GD) 16 – 18 and lactation day (LD) 8 – 10, urine samples within 24 h from pregnant or postpartum mice were collected. On another successive 3 days (day 1 – day 3), urine samples within 24 h from non-pregnant mice were collected. Data are expressed as mean ± SEM, n = 5. The concentration of L-Car (B) and mRNA levels of carnitine transporters (C) in the kidney of non-pregnant and pregnant mice at GD 18. Data are expressed as mean ± SEM, n = 10. Compared with the non-pregnant group, $^{***}P < 0.001$.

Fig. 4 The effect of E2 (A), P4 (B), 10 μM CORT and cortisol (C) on the mRNA expression of OCTN2 in HK-2 cells, and the concentration of L-Car in HK-2 cells in response to P4 (D) treatment. Cells were incubated with or without designated concentrations of hormones for 24 h. Data are expressed as mean ± SEM from two independent experiments conducted in triplicate. Compared with the control, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Fig. 5 The mRNA expression of PPARα (A) and PPARγ (B) in HK-2 cells in response to P4 treatment, and the effect of WY14643 (100 μM) on 20 μM P4-induced down-regulation of OCTN2 (C) and PPARα (D). Data are expressed as mean ± SEM from two independent experiments conducted in triplicate. Compared with the control, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Compared with the 20 μM P4 group, $^{\#\#\#}P < 0.001$. (E) The mRNA level of Pparα in the kidney of non-pregnant and pregnant mice at GD 18. Data are expressed as mean ± SEM, n = 10. Compared with the non-pregnant group, $*P < 0.05$.

Fig. 6 The effect of E2 (A), P4 (B), 100 μM CORT and cortisol (C) on the d_3-L-Car accumulation in MDCK-hOCTN2 cells. Cells were incubated with 3 μM d_3-L-Car in the absence or presence of designated concentrations of hormones for 3 min. (D) The effect of P4 on the d_3-L-Car accumulation in HK-2 cells. Cells were incubated with 10 μM d_3-L-Car in the absence or presence of L-Car (positive control) or P4 for 10 min. Uptake is expressed as the percentage of d_3-L-Car accumulation without inhibitors. Data are expressed as mean ± SEM from two independent experiments conducted in triplicate. Compared with the control, $^{***}P < 0.001$.

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Fig.7 The concentration of L-Car in the liver (A); the concentration of β-hydroxybutyrate in the liver (B) and serum (C); the mRNA levels of key enzymes involved in fatty acid β-oxidation (D) and β-hydroxybutyrate synthesis (E) in the liver of non-pregnant and pregnant mice at GD 18. Data are expressed as mean ± SEM, n = 10. Compared with the non-pregnant group, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

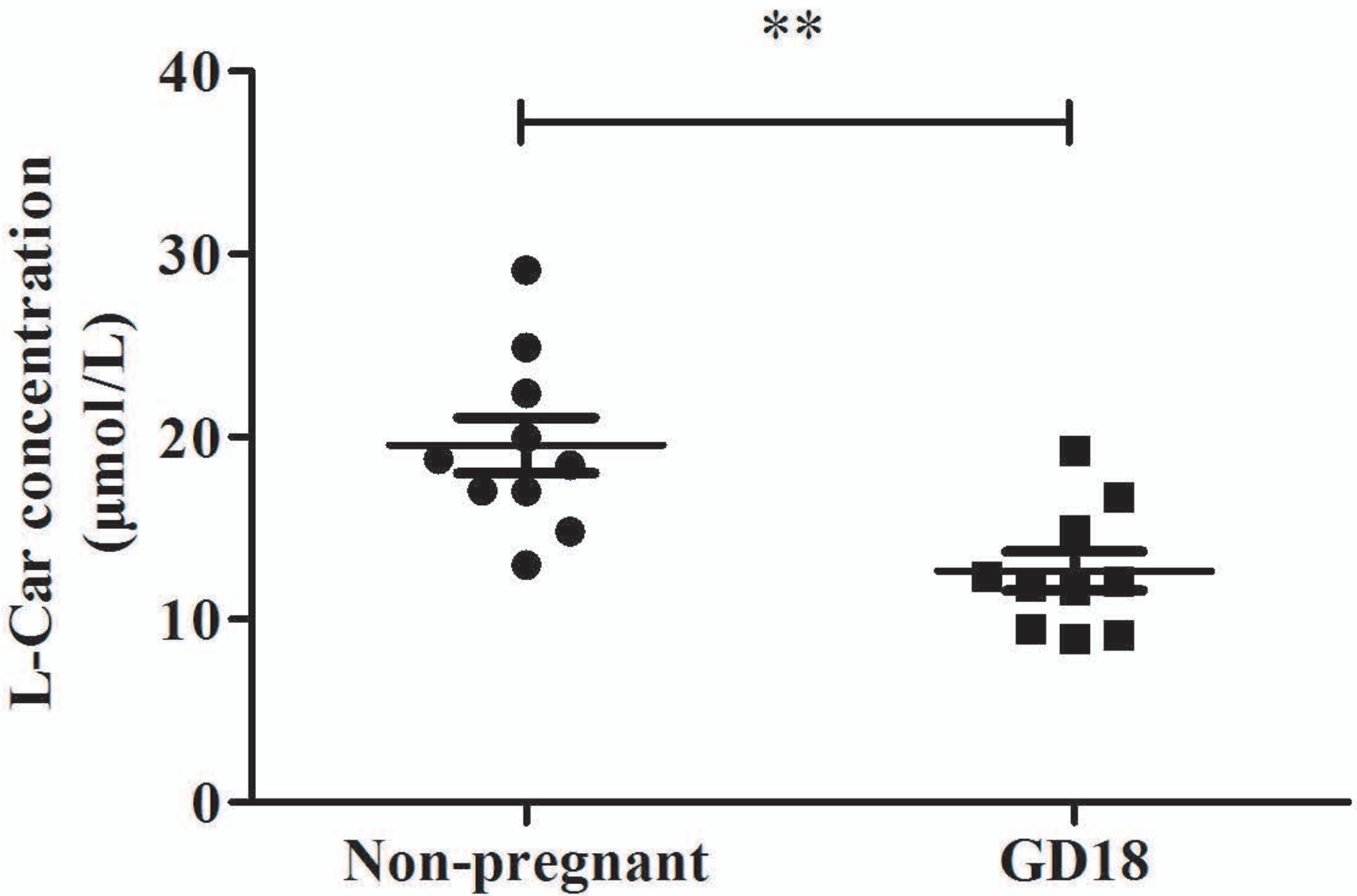

Figure 1

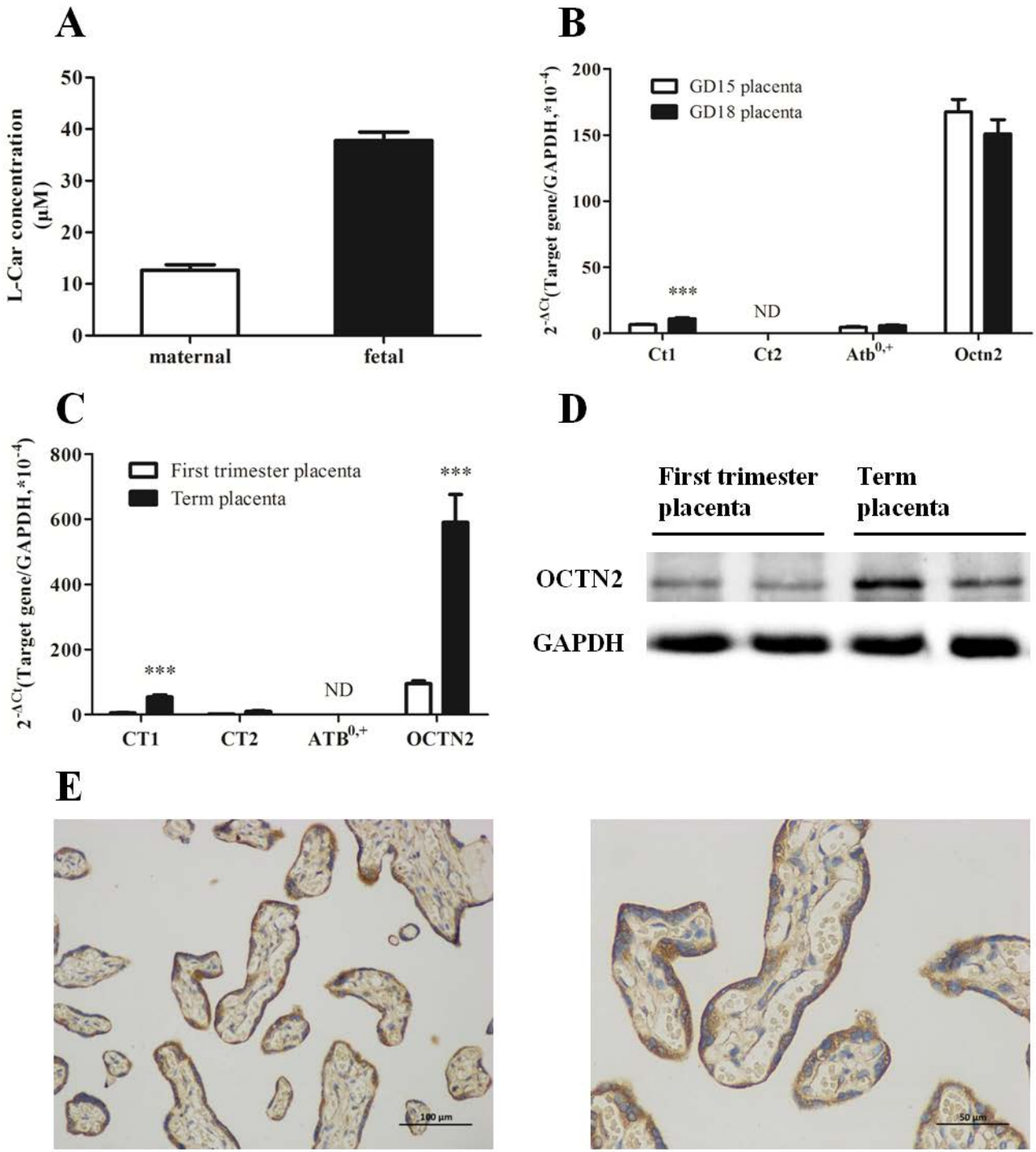
A
L-Car concentration (μM)
50
40
30
20
10
0
maternal
fetal
B
GD15 placenta
GD18 placenta
2-ΔCt(Target gene/GAPDH,*10-4)
200
150
100
50
0
***
ND
Ct1
Ct2
Atb0,+
Octn2
C
First trimester placenta
Term placenta
2-ΔCt(Target gene/GAPDH,*10-4)
800
600
400
200
0
***
***
ND
CT1
CT2
ATB0,+
OCTN2
D
First trimester placenta
Term placenta
OCTN2
GAPDH
E
100 μm
50 μm

Figure 2

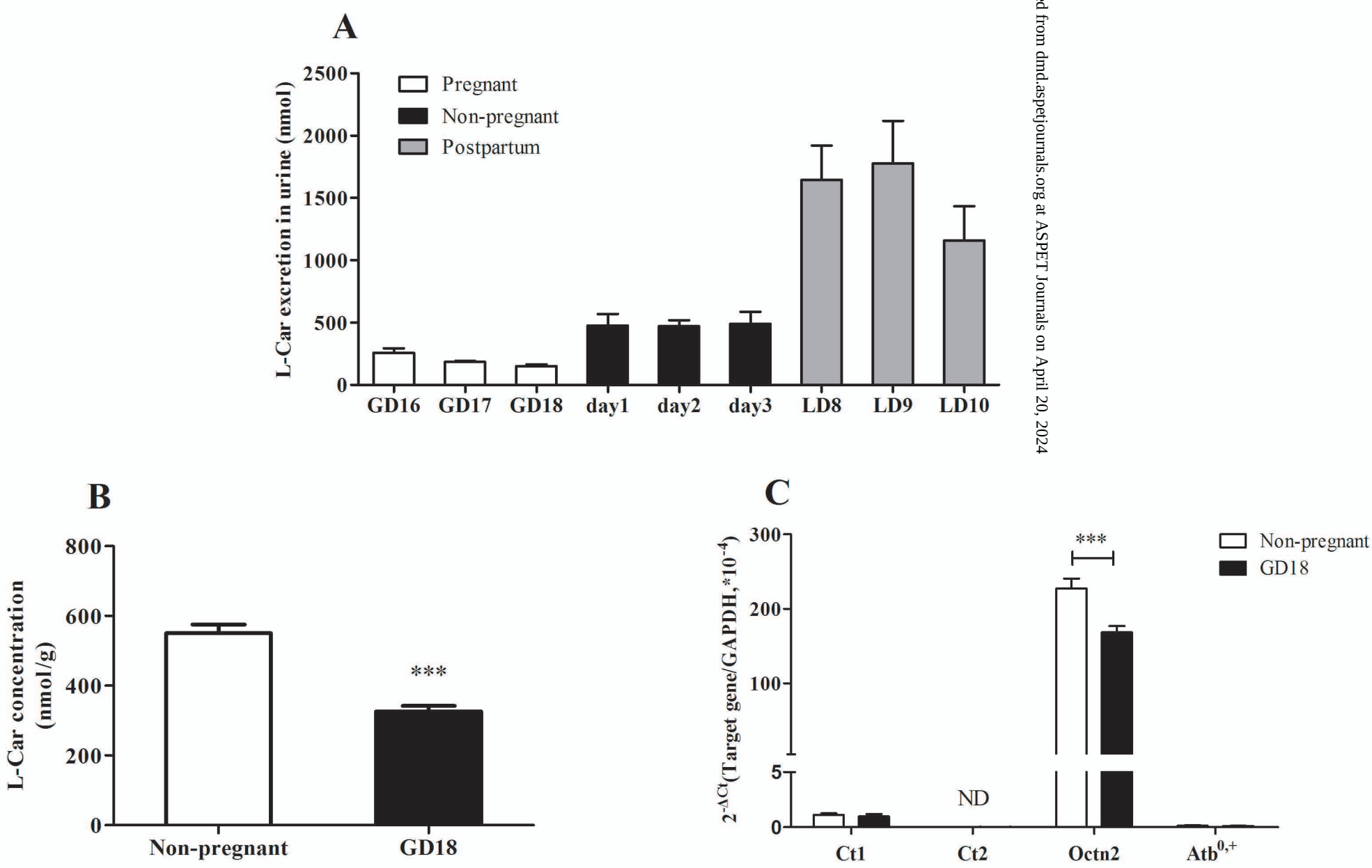

Figure 3

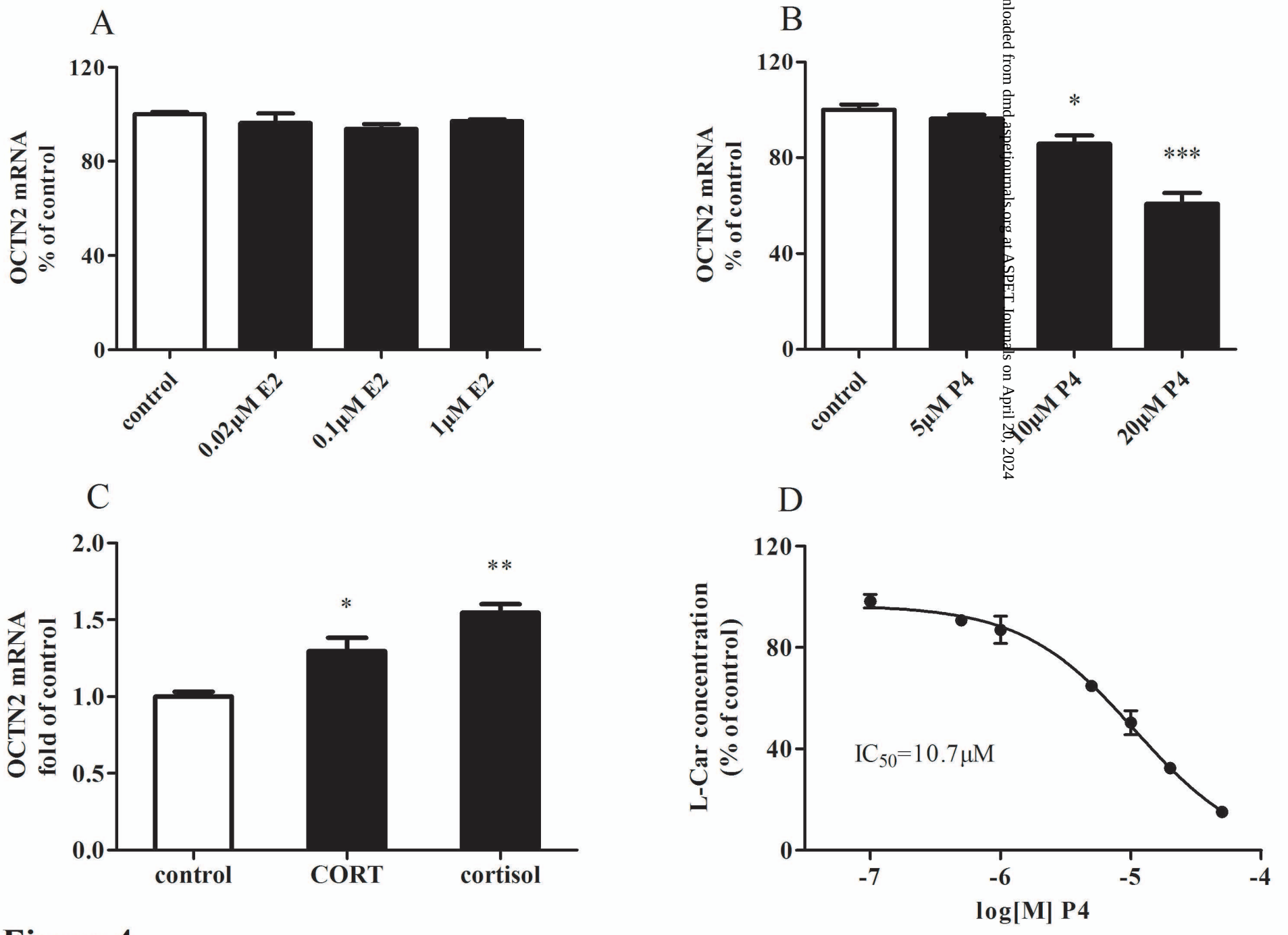

Figure 4

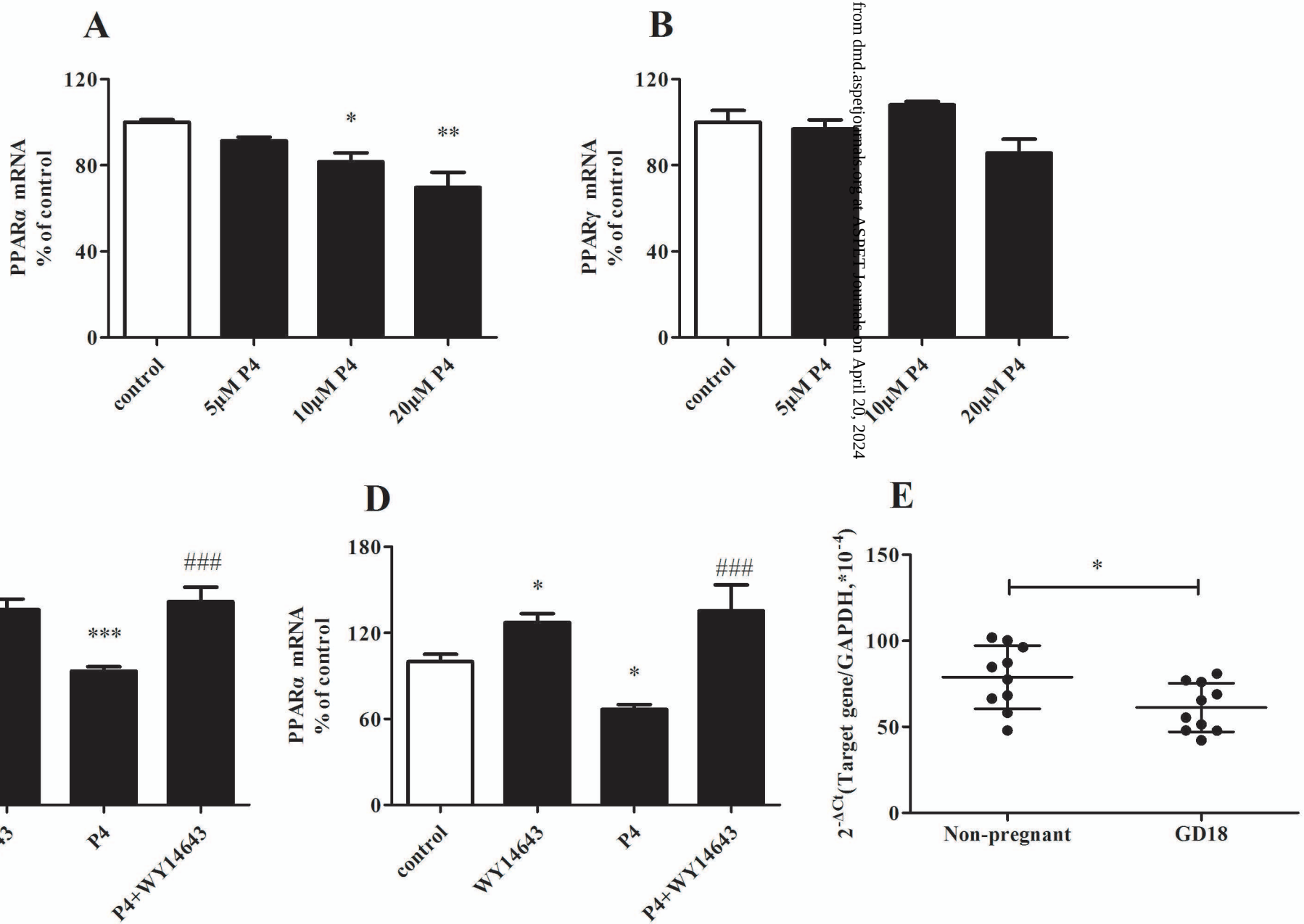

Figure 5

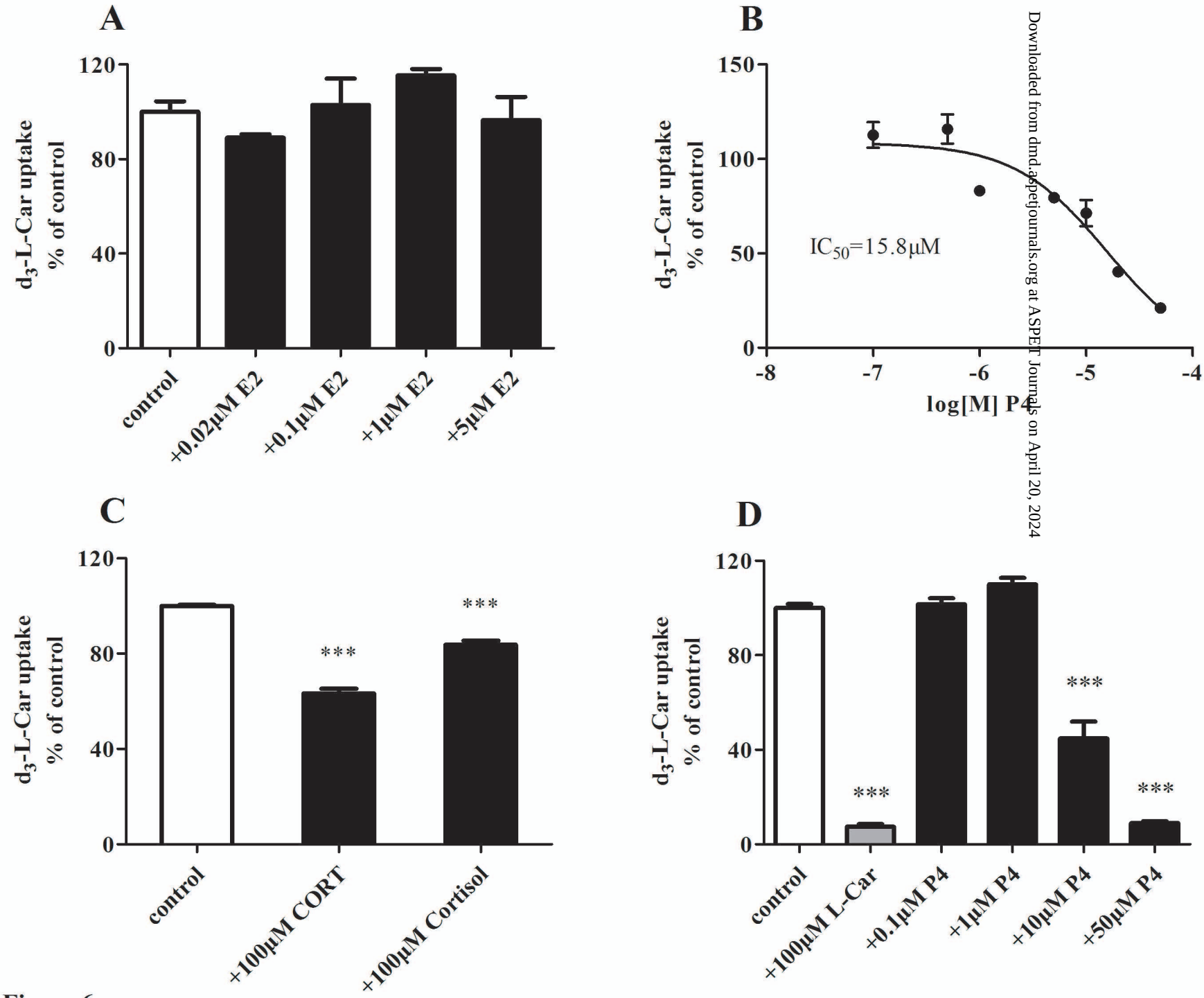

Figure 6

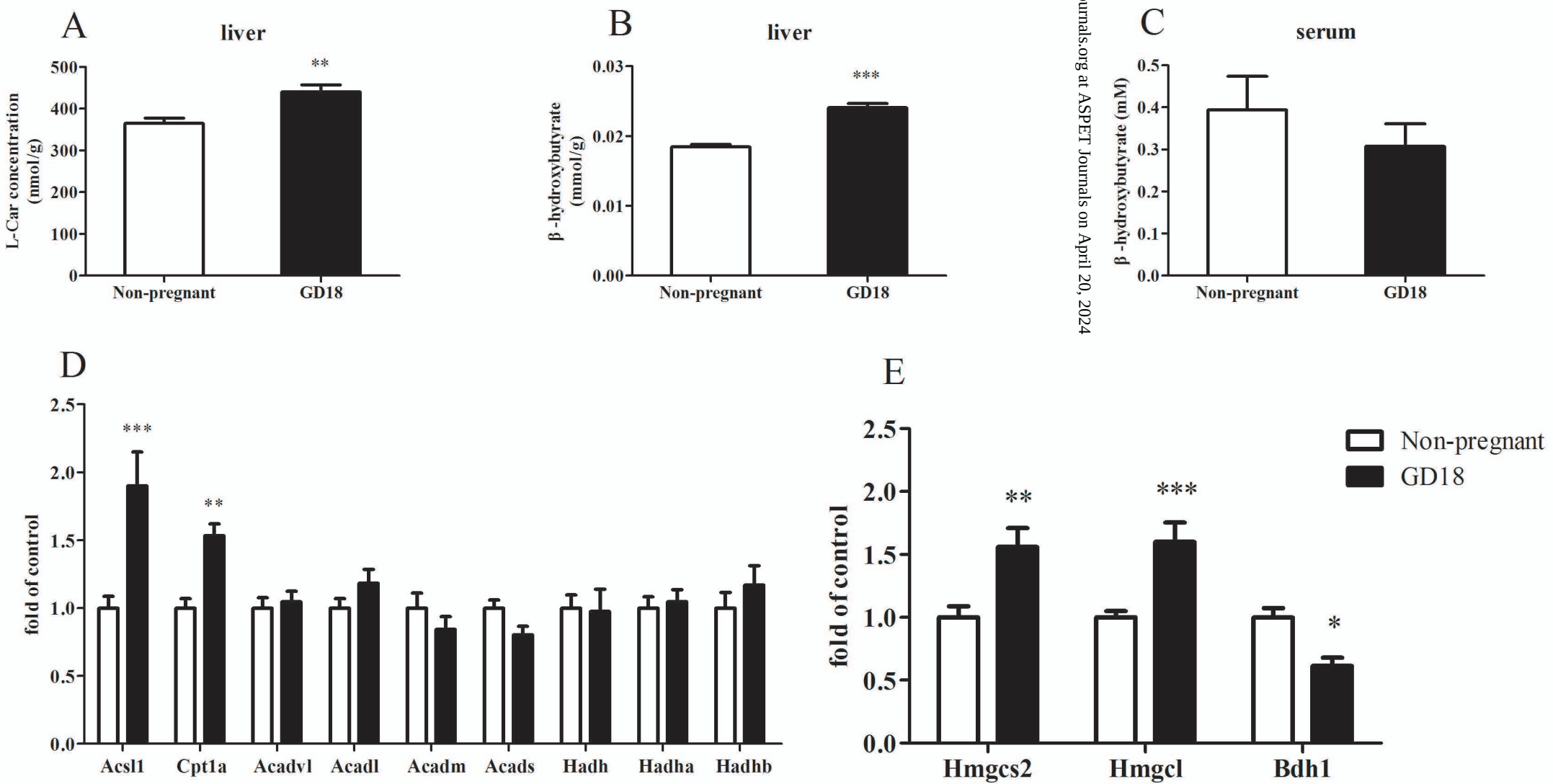

Figure 7

Drug Metabolism and Disposition

Supplementary Information

Maternal plasma L-carnitine reduction during pregnancy is mainly attributed to OCTN2 mediated placental uptake and does not result in maternal hepatic fatty acid β-oxidation decline

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Supplementary figures and tables

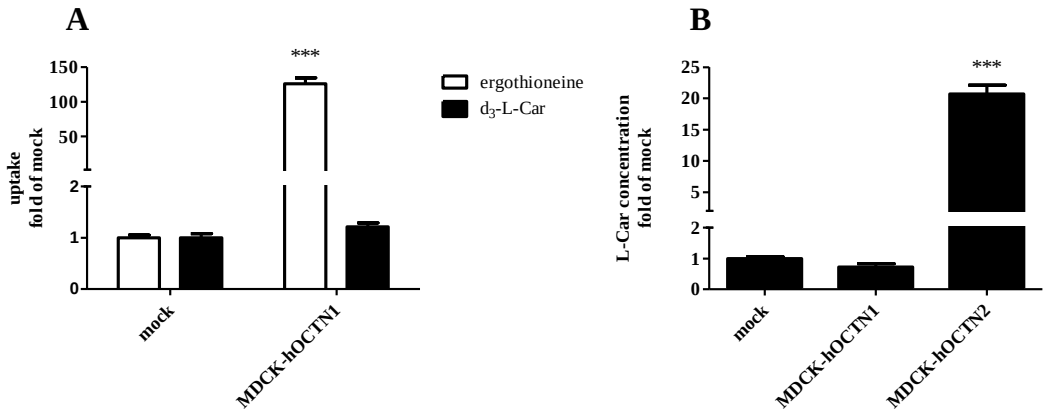

Fig. S1 (A) The accumulation of ergothioneine and d_3-L-Car in MDCK-hOCTN1 and mock cells. (B) The concentration of L-Car in MDCK-hOCTN1, MDCK-hOCTN2 and mock cells. Data are expressed as mean ± SEM from two independent experiments conducted in triplicate. Compared with the accumulation in mock cells, ***$P < 0.001$.

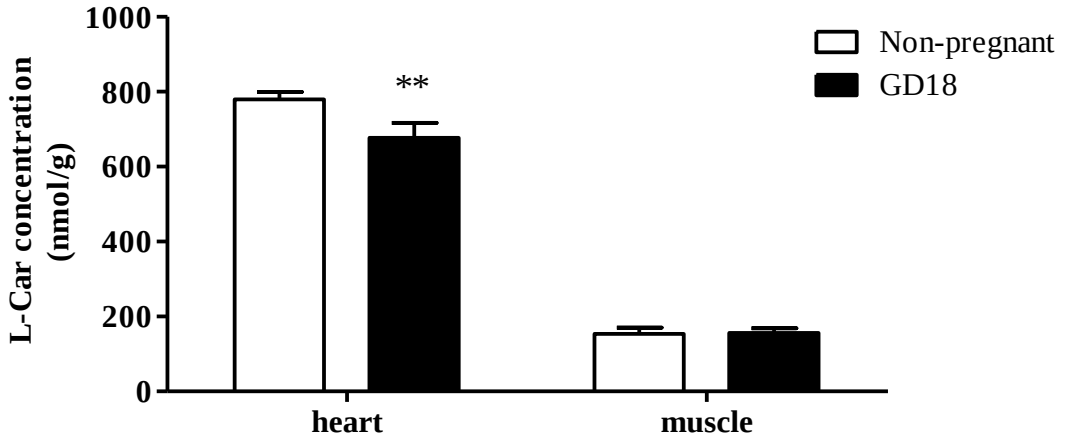

Fig. S2 The concentrations of L-Car in the heart and muscle of non-pregnant and pregnant mice at gestation day (GD) 18. Data are expressed as mean ± SEM, n = 10. Compared with the non-pregnant group, **$P < 0.01$.

Supplemental Table 1

Primers used in quantitative real-time polymerase chain reaction (qRT-PCR)

Gene	accession number	Direction	Sequence(5→3)	Product Length (bp)
Ct1	NM_001039371.2	Forward	GGCCTTTGTCTTGCTAAATGA	70
		Reverse	GAGACCACCAATCGATCCTG	
Ct2	NM_027572.1	Forward	GTCCTCGCTCACCTGTATGC	65
		Reverse	CCATCCTTTTCGCAGTCTTG	
Octn2	NM_011396.3	Forward	CACGATGGCTCATCTCTCAA	73
		Reverse	CCATTGATTTTGGCAGCTTT	
$Atb^{0,+}$	NM_020049.4	Forward	GAAGTTTTTCGGCCAGGAC	78
		Reverse	ATCCATTCCACTTGACCTCTG	
Pparα	NM_011144.6	Forward	CACGCATGTGAAGGCTGTAA	78
		Reverse	GCTCCGATCACACTTGTCG	
Acsl1	NM_007981.4	Forward	TGCCAGAGCTGATTGACATTC	101
		Reverse	GGCATACCAGAAGGTGGTGAG	
Cpt1a	NM_013495.2	Forward	GACTCCGCTCGCTCATTC	62
		Reverse	TCTGCCATCTTGAGTGGTGA	
Acadvl	NM_017366.3	Forward	GGTGGTTTGGGCCTCTCTA	82
		Reverse	GGGTAACGCTAACACCAAGG	
Acadl	NM_007381.4	Forward	GCTTATGAATGTGTGCAACTCC	81
		Reverse	CCGAGCATCCACGTAAGC	
Acadm	NM_007382.5	Forward	AGTACCCTGTGGAGAAGCTGAT	100
		Reverse	TCAATGTGCTCACGAGCTATG	
Acads	NM_007383.3	Forward	AGGTCCTGGAGGTCTGTGC	86
		Reverse	CAGTCCCGAACACCGAGA	
Hadh	NM_008212.4	Forward	TGGATACTACAAAGTTCATCTTGGA	75
		Reverse	AAGGACTGGGCTGAAATAAGG	
Hadha	NM_178878.2	Forward	TTCTTAAAGACACCACAGTGACG	80
		Reverse	CTTCTTCACTTTGTCGTTCAGC	
Hadhb	NM_145558.2	Forward	TGAAAACAAGCAATGTGGCTA	95
		Reverse	TGAAGAGATACAAGCCATGGTG	
Hmgcs2	NM_008256.4	Forward	ATACCACCAACGCCTGTTATGG	111
		Reverse	CAATGTCACCACAGACCACCAG	
Hmgcl	NM_008254.3	Forward	ACTACCCAGTCCTGACTCCAA	123
		Reverse	TAGAGCAGTTCGCGTTCTTCC	
Bdh1	NM_175177.4	Forward	GCTTCCTTGTATTTGCTGGC	130
		Reverse	TTCTCCACCTCTTCACTGTTG	
Gapdh	NM_008084.2	Forward	TGTGTCCGTCGTGGATCTGA	150
		Reverse	TTGCTGTTGAAGTCGCAGGAG	
CT1	NM_018420.2	Forward	TTGTCTTATTGTAATGTTTCTTCCAGA	112

		Reverse	GTTAAAGGCAGCACTGATGGT	
CT2	NM_033125.2	Forward	GCTGAGCTGTATCCAACCATT	104
		Reverse	CTGCTGAGGTCCACAGAGAAC	
OCTN2	NM_003060.3	Forward	GCAGCATCCTGTCTCCCTAC	91
		Reverse	GCTGTCAGGATGGTCAGACTT	
$ATB^{0,+}$	NM_007231.5	Forward	TGAAATGCCCGAGTTTCTTC	61
		Reverse	TTCTCTGATGAAGCCGACACT	
PPARα	NM_005036.5	Forward	GCTTTGGCTTTACGGAATACCA	77
		Reverse	TGAAAGCGTGTCCGTGATGA	
PPARγ	NM_005037.5	Forward	ATCTCTCCGTAATGGAAGACCACTC	488
		Reverse	CCCAAACCTGATGGCATTATGAGAC	
GAPDH	NM_002046.7	Forward	GCACCGTCAAGGCTGAGAAC	138
		Reverse	TGGTGAAGACGCCAGTGGA	