1 **An high dose of a conjugated linoleic acid increases fatty liver and insulin**

2 **resistance in lactating mice**

3 Kun Pang[1], Song bo Zhu [2], Liqiang Han [3*]

4 1 College of Animal Science and Veterinary Medicine, Xinyang Agriculture and

5 Forestry University, Xinyang 464006,China

6 2 Zhengzhou Academy of agriculture and Forestry Sciences, Zhengzhou 450005,

7 China

8 3 College of Animal Science and Veterinary Medicine, Henan Agricultural University,

9 Zhengzhou 450002, P.R .China

10 *Correspondence :Liqiang Han

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12 Short title: a high dose of CLA led to impaired lactation performance

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20 **Abstract**

21 This study aimed to evaluate the effect of a high dose of conjugated linoleic acid

22 (CLA) on lactating mice. In experiment one, KUNMING mice were separated into

23   four groups (n = 6 per group); the control (CON) group received 3.0% linoleic acid

24   oil (LA), the L-CLA group received 1.0% LA and 2.0% CLA mixture, the M-CLA

25   group received 2.0% LA and 1.0% CLA mixture, and the H-CLA group received

26   3.0% CLA mixture. Feeding proceeded from day 4 to day 10 during lactation. In

27   experiment two, the CON group received 2.0% LA and the H-CLA group received

28   2.0% CLA. Blood parameters were analysed for all groups, and insulin tolerance tests

29   (ITTs) were conducted. CLA treatment did not affect the dam weight, but it

30   significantly decreased the food intake of dams. Furthermore, CLA decreased the

31   weight of pups, which was attributed to lower milk fat. H-CLA group mice displayed

32   increased liver weight and liver triglyceride content, as well as a higher TG content

33   and γ-GT activity in blood. Moreover, a high dose of CLA resulted in insulin

34   resistance, possibly affecting the RBC and HCB of blood. In conclusion, lactating

35   mice receiving a high dose of CLA led to fatty liver, insulin resistance, and impaired

36   lactation performance.

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38   ***Keywords***: mouse; CLA; fatty liver; insulin resistance; lactation

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42   **Introduction**

43   Conjugated linoleic acids (CLAs) are molecules mostly found in meat and dairy

44   products. CLAs have emerged as a possible adjuvant treatment for obesity because

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45   some studies show a reduction in body fat and increased lean mass after

46   supplementation of a mixture of two isomers in the diet [1,2]. CLAs are also reported

47   to affect carcinogenesis, glucose and lipid metabolism, diabetes, body composition,

48   and immune cell functions [3-5]. However, certain isomers seem to cause fat

49   accumulation in the enlarged liver of mice [6], and can induce insulin resistance in

50   mice and humans [7,8].

51   In humans and other animals, milk lipids are markedly affected by the lipid

52   composition of the diet, the maternal energy balance, and metabolism. Maternal lipid

53   intake during lactation can alter milk composition, which in turn can affect the growth

54   of the litter [9] and change lipid metabolism and liver enzymes [10]. Many previous

55   studies showed that CLAs can lower triacylglycerol concentrations and alter the fatty

56   acid profile in the milk of lactating animals including human, mouse, rat, sheep, goat

57   and cow [11-17]. Feeding lactating mice with CLA can result in diminished

58   mammary gland lipogenesis and reduced growth rate [18,19], but the exact metabolic

59   and physiological changes involved remain unclear. More recently, Bezan (2018)

60   demonstrated that feeding a high dose (3%) of CLA increases insulin resistance and

61   exacerbates hepatic steatosis in male Wistar rats [20]. The intake of trans-fatty acids

62   during lactation was also found to increase maternal lipids in the liver (Assumpção,

63   2004) and liver weight [21]. As indicated above, it is most likely that CLA not only

64   affects mammary glands during lactation, but also liver and glucose metabolism.

65   CLAs used in supplements originate from the processing of vegetable oils such

66   as Safflower oil, and always include an equimolar mixture of c9,t11-CLA and

67   t10,c-12-CLA. Most studies of CLAs have used a mixture of isomers, with

68   c9,t11-CLA and t10,c-12-CLA the most abundant [20,22]. To explore this further,

69   two experiments were performed in which mice were fed with a CLA mixture. Firstly,

70   the effects of different doses of the CLA mixture on the milk and liver of dams was

71   investigated. Secondly, we tested whether a high dose of CLA could cause insulin

72   resistance in dams.

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Materials and Methods

Diets and feeding

76   Kunming (KM) mice were approved for use in this study by the University of

77   Zhengzhou for Medical Sciences Animal Care and Use Committee. Animals were

78   housed at 24°C with 40% humidity and a 12/12 h light-dark cycle. Animals were

79   provided with food and water random. A mouse-breeder diet (Laboratory Animal

80   Central, Henan province, China) containing the required complement of nutrients for

81   lactation was used as a carrier for each of the four fatty acid treatments. The fatty acid

82   composition of CLA oil and LA oil is shown in Table 1. Diets were prepared the day

83   before parturition and stored at 4°C.

84   In experiment one, all dams were fed the laboratory chow diet for the first 3 days

85   of lactation. On day 4 postpartum, six lactating mice were randomly assigned to the

86   control group (CON group, 3.0% LA oil), the low level CLA group (L-CLA group,

87   1.0% CLA + 2.0% LA), the median level CLA group (M-CLA group, 2.0% CLA +

88   1.0% LA), and the high level CLA group (H-CLA group, 3.0% CLA) from day 4 to

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89   10 postpartum. The litter number was normalised to eight at postpartum. Dam weight,

90   food intake, and the eight pups weight were determined daily between days 4 to 10

91   postpartum. The accurate assessment of food intake was achieved by accurately

92   collecting spilled food from cages. Based on the previous day's intake, a sufficient

93   amount of feed was offered to ensure at least 10% refusal. On day 10 postpartum,

94   pups were separated from dams for 2 h before feeding to ensure maximal

95   accumulation of milk in the mammary gland. Subsequently, all dams were milked by

96   suction. Pups were killed by cervical dislocation, and their abdomens were opened

97   and milk clots within the stomach were weighed and stored at -78°C until analysis

98   [12,23]. The milk lipid concentration was measured gravimetrically after

99   chloroform-methanol extraction by a modified Folch method [24]. Protein

100   concentration was determined by the Biuret method with casein as a standard. The

101   milk lactose concentration was estimated by Cocciardi method [25]. Dams were killed

102   on day 10 of lactation. Mammary and liver tissue from lactating mice was excised and

103   weighed, frozen in liquid nitrogen, and stored at -80°C. Triglycerides in liver were

104   extracted and analysed using a Tissue Triglyceride Assay Kit (E1013, Applygen

105   Technologies Inc) according to the manufacturer's protocol.

106   In experiment two, dams received 3.0% LA oil (CON group) or 3.0% CLA oil

107   (H-CLA group) from day 4 to day 10 of lactation. Feeding conditions were the same

108   as in the first experiment. Blood was collected from dams and analysed in the First

109   Affiliated Hospital of Zhengzhou University. Plasma insulin concentrations were

110   quantified using a Mouse Insulin Ultra-sensitive ELISA Kit (BC1710, Solarbio). For

111   ITTs, on the 11th day of lactation, all pads were renewed, and dams were starved for

112   6 h and weighed. Each dam was intraperitoneally injected with insulin (Novolin) at a

113   dose of 0.75 U/kg body weight. Before injection, the baseline blood glucose

114   concentration was measured and recorded as 0 min. After injection of insulin, the

115   blood glucose level in the tail vein was measured at 15, 30, 60, 90 and 120 min using

116   a TIDEX glucose analyser (Sankyo, Tokyo). The rate constant for each ITT (KITT)

117   was calculated using the equation KITT (%/min) = 0.693 / t(1/2), where t(1/2) was

118   calculated from the slope of plasma glucose concentration during 15−90 min after

119   administration of intravenous insulin.

120   **Statistical analysis**

121   Results are reported as mean ± standard deviation (SD). Data statistics and

122   differences among groups were analysed by SPSS 13.0 (IBM, USA). All experiments

123   were conducted in triplicate and repeated at least twice. Data were analysed for

124   significant differences using a general linear model of repeated measures, one-way

125   analysis of variance (ANOVA), or Student's t-tests. A p-value <0.05 was considered

126   statistically significant.

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128   **Results**

129   **Dam body weight and food intake**

130   Dam body weight increased progressively, and revealed a linear effect over time

131   (p <0.01, Fig. 1A), but there were no significant differences between different

132   treatment groups. Significant effects of treatment and time were observed for food

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133   intake of dams (p <0.05, Fig. 1B). Within the CLA treatment groups, there was a

134   significant difference in food intake between the L-CLA and M-CLA groups, and the

135   H-CLA group.

136   **Pup body weight**

137   The eight pups weight was measured daily and the results are shown in Fig. 2.

138   Significant effects for both both time and treatment were observed for pup weight

139   gain over the feeding period. Although the pup weight in all four groups increased

140   from day 4 to day 10, pups in the H-CLA group grew more slowly, and at the end of

141   the study, the eight pup weight of the H-CLA group was significantly lower than that

142   of the other groups (39.38 vs. 54.47, 54.95, and 51.13, p <0.01) by day 10 of

143   lactation.

144   **Milk and liver**

145   The milk clot weight obtained from the stomach ranged between 0.91 g and 0.56

146   g/suckling pup. The milk fat content of the H-CLA group was significantly lower than

147   that of the other groups. There were no significant differences in protein and lactose

148   concentrations in milk between CLA and control groups (Table 2). Similarly, there

149   were no differences in the weight of mammary tissue between these groups. Liver

150   weight was increased gradually with increasing dose of CLA, and there was a

151   significantly higher TG content in the liver of H-CLA group mice compared with

152   other groups (Table 2).

153   **Blood metabolite analysis**

154   Similar to experiment one, in experiment two, the weight of pups in the high

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155   dose CLA group was significantly lower than that in the control group (Fig. 3). CHO,

156   HDL and LDL levels in serum were unaffected, but plasma T and γ -GT activity in

157   the H-CLA treatment group were higher than in the control group (p <0.05), and

158   glucose was also increased (p = 0.07, Table 3). Insulin activity was significantly

159   higher in the H-CLA group. Interestingly, CLA feeding resulted in higher RBC and

160   HGB values in the blood of mice in the H-CLA group.

161   **Insulin tolerance testing**

162   At 0 min, the blood glucose level in the control group was 6.62 mmol/L, and that of

163   the CLA group was 7.04 mmol/L (Fig. 3). After injection of insulin, glucose in the

164   control group decreased to 4.03 mmol/L at 90 min, while glucose levels in the H-

165   CLA group were not obviously decreased (6.08 mmol/L). As shown by the decrease

166   in KITT, feeding mice with an high dose of CLA resulted in a significant insulin

167   resistance state.

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169   **Discussion**

170   CLA can reduce fat deposition, weight gain, and food/energy intake in mice [26].

171   In our present study, CLA treatment decreased food intake but did not reduce dam

172   weight. Although the mechanism is not clear, it is likely related to the lactation stage,

173   since mammary glands are the most active lipid-secreting organ in lactating mice [27].

174   Chin (1994) reported that increased maternal intake of CLA during lactation

175   enhanced the growth rate of suckling neonatal rats [28]. In contrast to the stimulatory

176   effect of CLA, mixtures of CLA [19] or isoforms [18] caused litter pups to grow

177   slowly or even die due to decreased milk fat. These contradictory results might be due

178   to differences in the content or isomeric distribution of the CLA supplement. In our

179   present study, 2% CLA diet fed to KM mice during lactation decreased both milk

180   weight and milk fat content. Milk is the only source of nutrients for suckling pups;

181   hence it was not surprising that a reduction in the fat and weight of milk were

182   accompanied by reduced pup weight during lactation. These results are consistent

183   with those of other studies on mice [12]. These findings confirmed that a high dose of

184   CLA lowered the content of milk fat and pup growth rate.

185   CLA has been reported to generate fatty liver, which could be a consequence of

186   increased lipogenesis in the liver to compensate the reduction in fat deposition in

187   adipose tissue [26]. We assessed the liver of dams and observed increased liver

188   weight and TG content in mice given a high dose of CLA. Kadegowda (2010)

189   reported that mice treated with CLA displayed decreased milk fat and increased liver

190   weight due to hepatic steatosis [29], similar to our current results. The effects of CLA

191   supplementation on mammary fat synthesis have received a great deal of attention

192   [30,31], but the potentially adverse effects of CLA on the liver and lipid metabolism

193   have been largely ignored in lactating animals. We suggest that severe lipid

194   accumulation occurs in the liver when lactating mice are given a high dose of CLA.

195   This was also confirmed by increased TG content and γ-glutamyl-transferase (γ-GT)

196   activity in the serum of the high CLA group.

197   There have been many reports regarding CLA causing insulin resistance [7]. In

198   the present study, ITTs clearly indicated that a high dose of CLA fed to mice caused

199   insulin resistance. In lactating mice, glucose is the main precursor for the synthesis of

200   lactose and triglyceride substrates. During the lactation stage, mammary glands have

201   increased demand for glucose, and are highly sensitive to insulin, while other organs

202   are relatively insensitive to insulin [32]. In this study, lactating mice fed with CLA

203   displayed aggravated insensitivity to insulin and exhibited insulin resistance,

204   implicating insulin as a key factor in CLA-mediated regulation of milk fat and

205   lactation.

206   Interesting, we observed for the first time that a high dose of CLA can result in

207   higher RBC and HGB levels in the blood of lactating mice. RBC and HGB are both

208   positively correlated with insulin resistance and NAFLD [33]. Thus, in the present

209   study, higher RBC and HGB may be a consequence of insulin resistance and fatty

210   liver in mice receiving an high dose of CLA.

211   In summary, a high level of CLA ingestion during lactation decreases milk fat

212   and increases lipid content in the liver. Therefore, CLA is a dietary factor with a

213   propensity to affect hepatic metabolism during lactation. Little is known about the

214   metabolic changes in animals during lactation, especially the possibility of a very

215   complex regulatory mechanism between lipid metabolism in the liver and mammary

216   lactation. The present findings provide a platform for further studies on the exact

217   mechanism and the safe use of CLA .

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219   **Conflict of Interest**

220   The authors declare no competing financial interests.

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313 **Table 1 composition of LA oil and CLA oil**

Composition	CLA（%）	LA（%）
Palmitic acid（C16：0）	4.89	3.58
Stearic acid（C18：0）	2.15	1.26
Oleic acid（C18：1）	10.64	11.21
Linoleic acid（C18：2）	0.75	80.13
Conjugated Linoleic acid（C18：2）	81.48	-
c9,t11 CLA	37.53	-
t10,c12 CLA	38.38	-
Others CLA	5.57	-
Linolenic acid（C18：3）	0	2.35
Others fatty acid	0.09	1.47

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315   **Table 2 Effect of CLA on the milk and liver of dams**

	CON group[#]	L-CLA group	M-CLA group	H-CLA group
Milk clotweight (g/pup)	0.89 ± 0.27^a	0.91 ± 0.12^a	0.85 ± 0.23^a	0.56 ± 0.29^b
Milk fat (mg/100mg)	15.42 ± 1.43^a	14.16 ± 1.96^a	13.37 ± 2.30^a	11.88 ± 1.99^b
Milk protein (mg/100mg)	18.79 ± 1.35	20.19 ± 2.30	17.96 ± 3.71	19.87 ± 3.32
Milk lactose (mg/100mg)	2.03 ± 0.56	1.89 ± 1.45	2.26 ± 0.85	1.93 ± 1.30
Mammary Weights (g)	3.88 ± 0.69	4.21 ± 0.79	4.39 ± 1.23	3.96 ± 1.06
liver Weights (g)	2.91 ± 0.29^a	3.02 ± 0.41^a	3.63 ± 0.55^{ab}	4.65 ± 0.59^b
TG of liver (mg/g tissue)	46.41 ± 27.07^a	94.51 ± 32.78^{ab}	92.5 ± 34.16^{ab}	230.14 ± 80.54^b

316 [a,b]Values followed by different letters in the same row indicate significant differences

317 (p <0.05). Values are means ± SD.

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318   [#]CON group, 342.0% LA oil; L-CLA group, 1.0% CLA + 2.0% LA; M-CLA group,

319   2.0% CLA + 1.0% LA; H-CLA group, 3.0% CLA.

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336   **Table 3 Effect of a high dose of CLA on the blood of dams**

Parameters	CON group[#]	H-CLA group	P-values
CHO（mmol/L）	2.52±0.38	2.86±1.01	0.461
TG（mmol/L）	1.04±0.34	2.17±1.26	0.044

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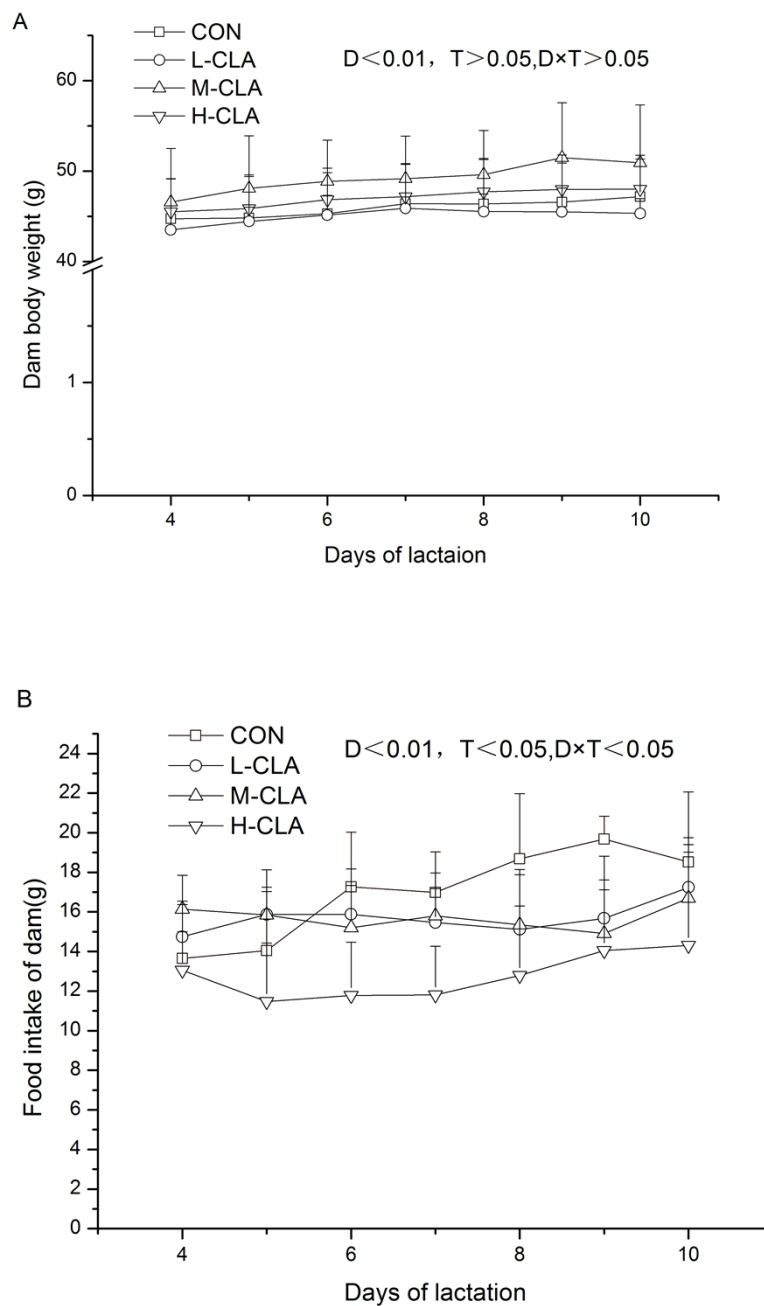|			
HDL（mmol/L）	1.53±0.21	1.56±0.46	0.887
LDL（mmol/L）	0.22±0.08	0.29±0.05	0.162
Glucose（mmol/L）	6.58±1.20	7.49±1.53	0.070
ALT（U/L）	39.2±10.51	41±27.38	0.896
AST（U/L）	69.4±13.97	74.16±7.28	0.560
γ-GT（U/L）	15.4±3.92	24.5±2.98	0.005
ALP（U/L）	22±8.02	28.5±2.29	0.183
Insulin (ng/ml)	6.38±1.72	27.15±4.91	＜0.001
WBC(10^9/L)	3.31±1.74	2.94±1.28	0.734
RBC(10^{12}/L)	7.35±0.47	8.66±0.34	0.002
PLT（10^9/L）	1415.83±84.93	1326±196.74	0.431
HGB(g/L)	117±8.10	140±4.04	0.002

337 # CON group, 3.0% LA oil; H-CLA group, 3.0% CLA oil.

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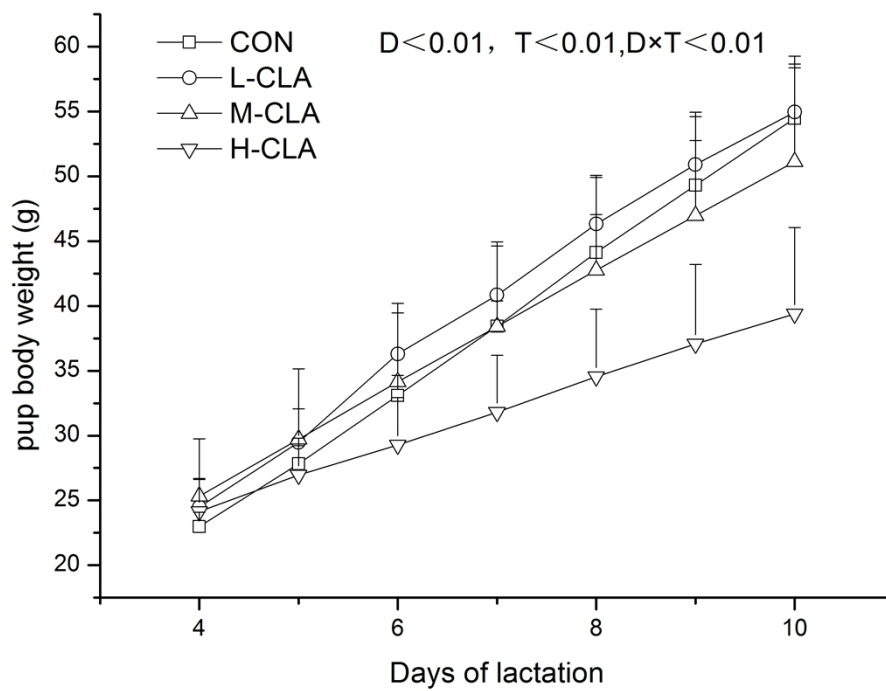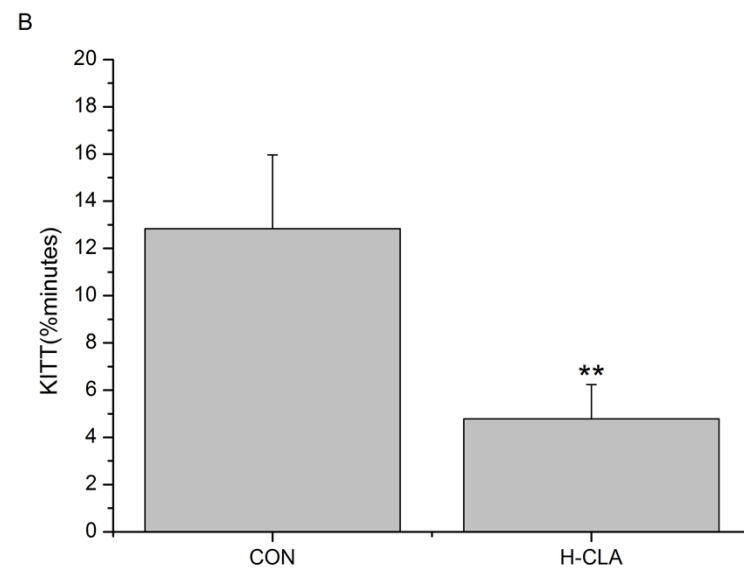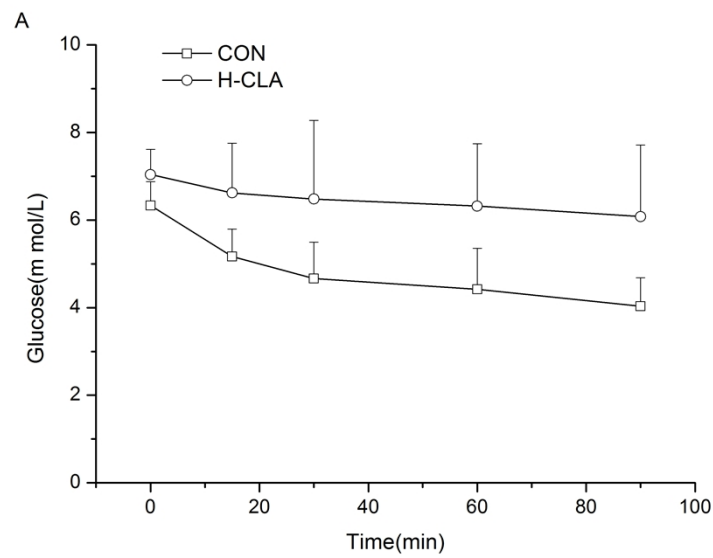339

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Figure 1

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350   **Figure 2**

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Figure 3

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374 **Figure 4**

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379   **Figure Legends**

380   **Figure 1.** Daily body weight and food intake of dams fed different doses of CLA.

381   Values are means ± SD (n = 6). (A) Dam body weight during day 4 to day 10 of

382   lactation. (B) Food intake during day 4 to day 10 of lactation. CON group, 342.0%

383   LA oil; L-CLA group, 1.0% CLA + 2.0% LA; M-CLA group, 2.0% CLA + 1.0% LA;

384   H-CLA group, 3.0% CLA; T, effect of CLA treatment. D×T, time and treatment

385   interaction.

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387   **Figure 2.** Daily weight of nursing pups fed different doses of CLA during day 4 to

388   day 10 of lactation. Values are means ± SD (n = 6). CON group, 342.0% LA oil;

389   L-CLA group, 1.0% CLA + 2.0% LA; M-CLA group, 2.0% CLA + 1.0% LA;

390   H-CLA group, 3.0% CLA; D, effect of lactation time, T, effect of CLA treatment;

391   D×T, time and treatment interaction.

392

393   **Figure 3.** Dams and eight pups of CON and H-CLA groups in experiment two at day

394   10 of lactation.

395

396   **Figure 4.** Insulin tolerance tests (ITTs) of lactating mice in CON and H-CLA groups.

397   Values are means ± SD (n = 6). (A) ITTs of lactating mice. Each dam was injected

398   with insulin at a dose of 0.75 U/kg body weight. Blood glucose level was measured at

399   0, 15, 30, 60, 90 and 120 min. (B) Calculation of KITT (%min) after a dose of 0.75 U

400   of insulin per kg of body weight. CON group, 2.0% LA oil; H-CLA group, 2.0% CLA;

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401   $**p < 0.01$.

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