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High-fat diet feeding modulates starvation and refeeding-induced acute hepatocellular injury and inflammatory response in mice

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Abstract

We previously reported that refeeding after a 48h fast, used as a model of starvation and refeeding, results in hepatic parenchymal cell injury. The objective of the present study was to address the effects of high-fat diet (HFD) feeding for a long period (94 days) on fasting and refeeding-induced hepatocellular injury and inflammatory response. Mice fed standard low-fat diet (LFD) or HFD for 94 days were fasted for 46h and then refed the same diet. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, and expression levels of genes involved in inflammation in abdominal white adipose tissue (WAT) and brown adipose tissue (BAT) were sequentially measured for first 20h after refeeding initiation. Refeeding after 46h of fasting resulted in a rapid and abnormal elevation in serum ALT and AST levels, which was greater in the LFD-fed mice than in the HFD-fed mice. The HFD-fed mice showed higher abdominal WAT mRNA of cell markers for eosinophils (Enpp3) and macrophages (Emr1, CD206) and several pro- and anti-inflammatory cytokines (IL-1α, IL-10, TGF-β) compared with the LFD-fed mice. In addition, the HFD-fed mice exhibited fasting and refeeding-induced increase in abdominal WAT mRNA of pro- and anti-inflammatory cytokines (IL-6, IL-1α, IL-1β and IL-10). Refeeding led to increased BAT mRNA of pro-inflammatory cytokines (IL-6, IL-18) in the HFD-fed mice. These results indicate that a fasting-refeeding regime in combination with HFD feeding attenuates acute hepatocellular injury but promotes abdominal WAT and BAT inflammatory response when compared with a fasting-refeeding regime alone.

Key words : adipose tissue, fasting, high-fat diet, inflammation, refeeding

Introduction

Unhealthy eating behaviors increase the risk of metabolic diseases. Acute starvation and refeeding is an important health problem that often arises by extreme dieting or occurs during natural disasters. A previous study demonstrated that refeeding with a standard LFD after a 48h fast, a study model of acute starvation and refeeding, induces a modest but significant elevation in the serum levels of ALT (an index of hepatocyte destruction)[1-3]. Patients with anorexia nervosa also exhibit mild to moderate elevation in their serum ALT and AST levels following refeeding therapy[4]. Several epidemiologic studies have reported that an increase in serum ALT levels is associated with the long-term development of multiple metabolic disorders and that this also applied to individuals with elevated serum ALT levels within the normal range[5,6].

In a previous study we also showed that a fasting-refeeding regime leads to increased liver inflammatory response[2]. It is well known that HFD accelerates the inflammatory response and the consequent induction of associated diseases[7] and that adipose tissue is an important site of inflammatory events in HFD-induced obesity[8,9]. They raise an interesting question regarding the effects of HFD feeding on fasting and refeeding-induced hepatocyte destruction and inflammation.

In this study, to investigate the effects of HFD feeding for a long period on fasting and refeeding-induced hepatocellular injury and inflammatory response, mice fed standard LFD or HFD for 94 days were fasted for 46h and then refed the same diet. We compared the serum ALT and AST levels, and expression levels of genes involved in pro- and anti-inflammatory response and tissue specific genes in the liver, abdominal WAT and BAT of these animals.

Materials and Methods

Diets

Casein, α-corn starch, sucrose, cellulose powder, AIN-76 mineral mixture[10], AIN-76 vitamin mixture[10], and choline bitartrate were purchased from Oriental Yeast (Tokyo, Japan). DL-methionine, soybean oil, lard, beef tallow and $NaNO_2$ were obtained from Wako Pure Chemical Ind. (Osaka, Japan). Purified powder standard LFD (5% fat, w/w) and HFD (21.5% fat) were prepared in our laboratory using food-grade ingredients. The composition of these diets is shown in Table 1.

Table 1. Composition of purified powder diets

	Standard low-fat diet (LFD) (g/kg)	High-fat diet (HFD) (g/kg)
α-Corn starch	550.75	385.75
Sucrose	100	100
Casein	200	200
DL-methionine	2.25	2.25
Soybean oil	50	50
Lard	0	82.5
Beef tallow	0	82.5
Mineral mixture1	35	35
Vitamin mixture1	10	10
Choline bitartrate	2	2
Cellulose	50	50

[1] AIN-76 mineral and vitamin mixtures (10).

Animals and experimental design

Specific-pathogen-free, 5-week-old female BALB/c mice were obtained from Charles River Japan (Atsugi, Japan). The animals were maintained on a commercial laboratory chow (Oriental Yeast) and were given water ad libitum. The non-purified diet comprised approximately 23.6% protein, 5.3% fat, 6.1% ash, 2.9% fiber and 54.4% nitrogen-free extracts. After an acclimatization period (5 days), the mice were separated into two different groups, that were 1) fed the standard LFD, 2) fed the HFD. Before starting the treatment with the test diets, body weights were comparable between the test groups. In all groups, a 12h/12h light/dark cycle was maintained and the room temperature was kept at 23±1℃. At the end of the feeding experiment of the test dietary groups for 94 days, the mice were deprived of food for 46h but were allowed free access to water. After this food

deprivation period, the animals were immediately administered the same diet ad libitum for 20h. The animals were sacrificed by decapitation at 0, 10 and 20h after refeeding had commenced, and their blood, livers, abdominal WAT and BAT were harvested. Blood was collected and allowed to clot for 1 hour at room temperature. Serum was then separated by centrifugation at 1,200 × g for 20 min at 4℃ and stored at -80℃ until analysis. Tissue samples were weighed and stored at -80℃ or in RNAlater (Thermo Fisher Scientific, Tokyo, Japan) at -20℃. The experimental procedures used in the present study confirmed to the guidelines of the Animal Usage Committee of Chiba University and Sagami Women's University.

Measurement of glucose, non-esterified fatty acid and insulin levels and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the serum

The serum glucose, non-esterified fatty acid, ALT and AST levels were measured using commercially available kits as follows: glucose CII-test, non-esterified fatty acid C, transaminase CII-test (Wako Pure Chemical Ind.). The serum insulin levels were measured using a mouse insulin ELISA kit (Morinaga Institute of Biological Science, Tokyo, Japan). All assays were performed in duplicate and the data averages were statistically analyzed.

RNA extraction and quantitative real-time RT-PCR

Total RNA was isolated from mouse livers and abdominal adipose tissues with an Isogen according to the manufacturer's instructions (Nippon gene co., Tokyo, Japan). The quantity and the quality of RNA samples were measured with a Nano Drop 1000 (Nanodrop Technologies, Wilmington, USA). Complementally DNAs were synthesized from these total RNA extracts using random primers and a reverse transcription system (Promega, Madison, WI, USA), according to the manufacturer's instructions. Real-time quantitative reverse transcriptase polymerase chain reaction (PCR) analyses were performed in 20μL of KAPA SYBR FAST qPCR Kit (Nippon Genetics Co, Ltd, Tokyo, Japan) containing 12

Table 2. Primer sequences (5'→ 3')

Gene	Acc. number	Forward	Reverse
Acsl1	BC056644	CGCTCACCACCTTCTGGTAT	ACCATCAGTGGTACCCGCTA
C-fos	NM_010234	CCTGTCCGGTTCCTTCTATG	AAGTAGTGCAGCCCGGAGTA
C-jun	NM_010591	ATGGGCACATCACCACTACA	GACACTGGGAAGCGTGTTCT
CD206	BC141338	TTGCCATGAAAACCGGAGTG	TCTGCCCAATGTTTGCACAC
COX2	NM_011198	CAAGACAGATCATAAGCGAGGA	GGCGCAGTTTATGTTGTCTGT
Emr1	U66888	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Enpp3	AY630402	CATGGAGTACCCTTTTGCCT	TGATTTGGGAAGGTTTTGGT
Hprt1	BC083145	AGGACTGAAAGACTTGCTCGAG	AATCCAGCAGGTCAGCAAAG
IL-1α	NM_010554	TCGGGAGGAGACGACTCTAA	TGAGTTTTGGTGTTTCTGGC
IL-1β	NM_008361	TCGCAGCAGCACATCAACAA	GGTGCTCATGTCCTCATCCT
Il1ra	M57525	GTGTGTTCTTGGGCATCCAC	CAGGACGGTCAGCCTCTAGT
IL-6	BC138766	TGATGCACTTGCAGAAAACA	ACCAGAGGAAATTTTCAATAGGC
IL-10	M84340	ATCGATTTCTCCCCTGTGAA	TGTCAAATTCATTCATGGCCT
IL-18	BC024384	GGCCGACTTCACTGTACAAC	CCTTCACAGAGAGGGTCACAG
Mpo	BC053912	CCGCCTGAACAATCAGTACC	ATTCAGTTTGGCTGGAGTGG
Ppargc1a	JX866948	GAAAGGGCCAAACAGAGAGA	GTAAATCACACGGCGCTCTT
Tgf-β	BC013738	GTCCTTGCCCTCTACAACCA	GTTGGACAACTGCTCCACCT
TLR5	AF186107	AACATCCAGGCGGCTGTC	GGACAGCGATCCCACCAC

pmole primers and 35 ng of reverse transcribed total RNA. The resulting amplified products were analyzed in real time using a Thermal Cycler Dice Real Time System TP870 (Takara Bio Inc., Tokyo, Japan). The primer sequences used are shown in Table 2. All PCR products were generated using a two-step protocol (95℃ for 5s, 60℃ for 30s) after an initial denaturation step (95℃ for 30s). The relative expression levels of the target gene products were calculated with the comparative threshold cycle method using Hprt1 as the normalization control.

Statistical analysis

All of the given data are the mean ± standard deviation (S.D.) of representative measurements. Statistical comparisons between measurements taken at 46h before (pre-fasting) and at 0, 10 or 20h after the commencement of refeeding were made using the unpaired Student's t test. Differences between the two dietary groups (the LFD and HFD groups) before fasting for 46h (prefasting) were also statistically analyzed using the unpaired Student's t test. Differences were considered significant at $P<0.05$. All statistical analyses were performed using SPSS software version 25 (SPSS Inc., Tokyo, Japan).

Results

Food intake and body, liver, abdominal white adipose tissue (WAT) and brown adipose tissue (BAT) weights

Mice fed the HFD for 94 days showed higher weights of whole body and abdominal WAT compared with mice fed the LFD for the same period (Prefasting, Fig.1B and D). In contrast, HFD feeding for 94 days had no significant effects on the liver and BAT weights (Prefasting, Fig.1C and E). No significant difference was observed between LFD and HFD groups in the food intake during the 0-20h period after refeeding (Fig. 1A). In both diet groups, body weight decreased by fasting for 46h, and then recovered to the prefasting levels at 10h after refeeding. The 46h fasting resulted in decreased liver and abdominal WAT weights in the LFD-fed mice, but not in the HFD-fed mice. The liver weight increased over the prefasting levels in both diet groups at 10 and 20h after refeeding. The BAT weight decreased in both diet groups by fasting, and then recovered to the prefasting levels in the LFD and HFD groups at 20 and 10h after refeeding, respectively.

Serum levels of glucose, insulin, nonesterified fatty acid, alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

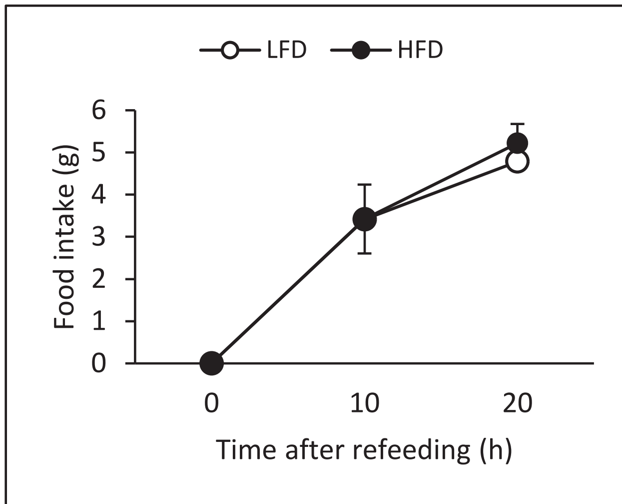
HFD feeding for 94 days had no significant effects on serum glucose, insulin, ALT and AST levels but resulted in elevated serum NEFA levels compared with LFD feeding (Prefasting, Fig.1F-J). Serum glucose and insulin levels rapidly and markedly decreased by fasting and then recovered to prefasting levels in both diet groups at 10h after refeeding. Fasting for 46h caused a transient increase in serum NEFA levels in the LFD-fed mice, but not in the HFD-fed mice. Hepatic parenchymal cell injury was predicted from the observed increases in serum ALT and AST levels in our subject mice. The 46h fasting was observed not to significantly affect serum ALT levels in both diet groups. Refeeding for 10 and 20h did however result in increased serum ALT levels in both diet groups, which was greater in the LFD group. Serum AST levels increased in the LFD group but were maintained at pre-fasting levels in the HFD group at 0-20h after refeeding.

Expression of specific genes in the liver

C-jun and C-fos are members of the immediate-early genes, and encode members of the leucine-zipper family of transcription factors[11]. These proteins regulate the cellular response genes after injury that are associated with tissue repair and cell apoptosis[12]. HFD feeding for 94 days had no significant effects on the liver expression levels of immediate-early genes (Prefasting, Fig.2A and B). Fasting and refeeding resulted in increased liver expression levels of immediate early genes (C-jun, C-fos) in both diet groups, which was greater in the LFD group.

Fig.1

A



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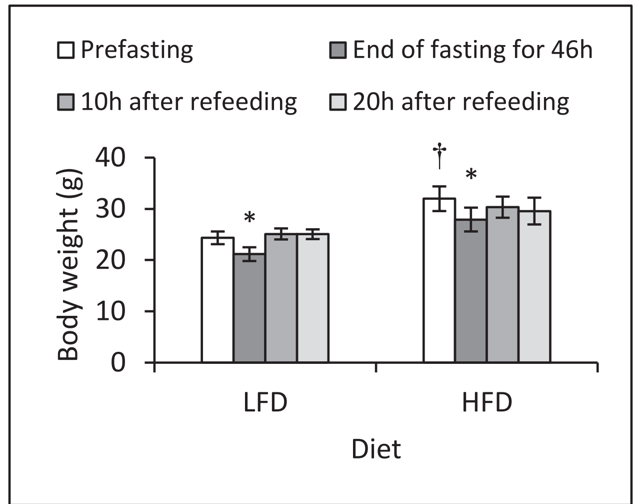

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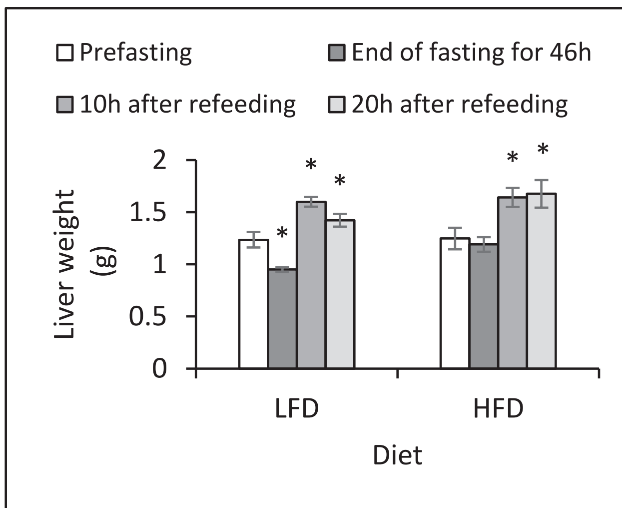

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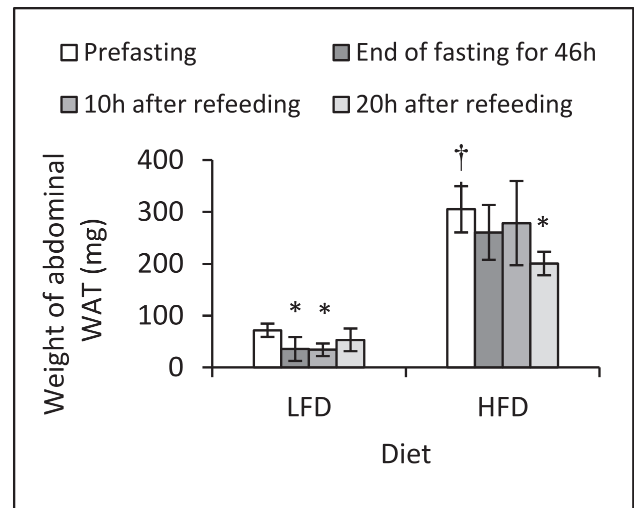

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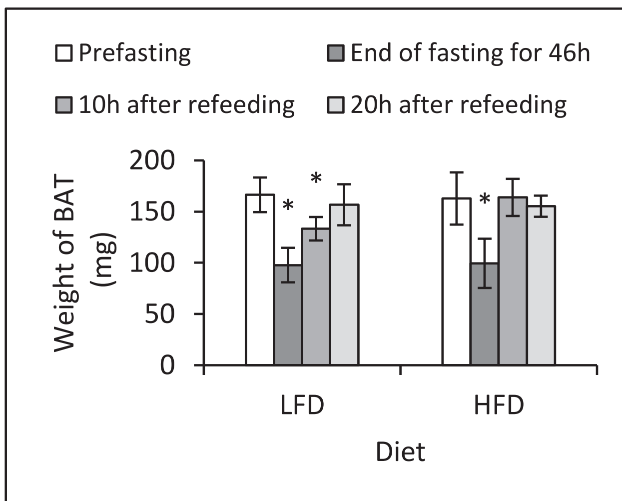

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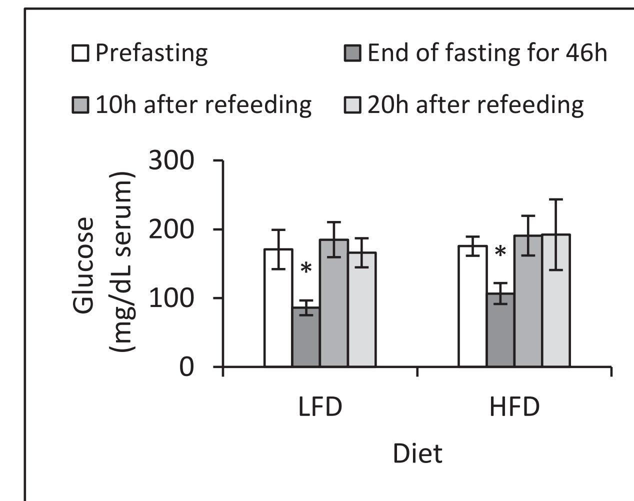
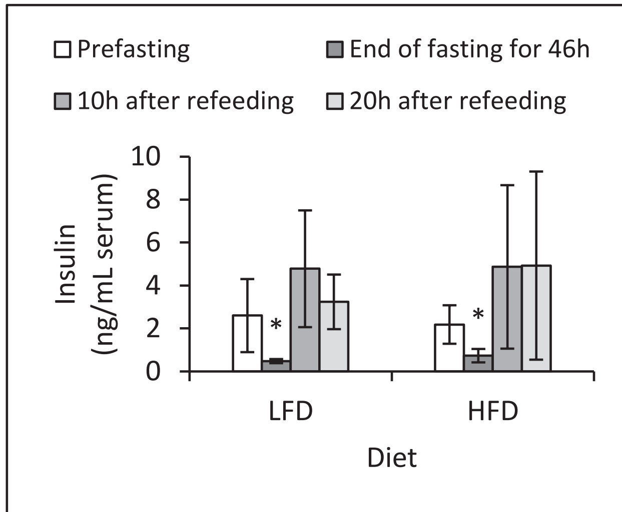


Fig.1

G



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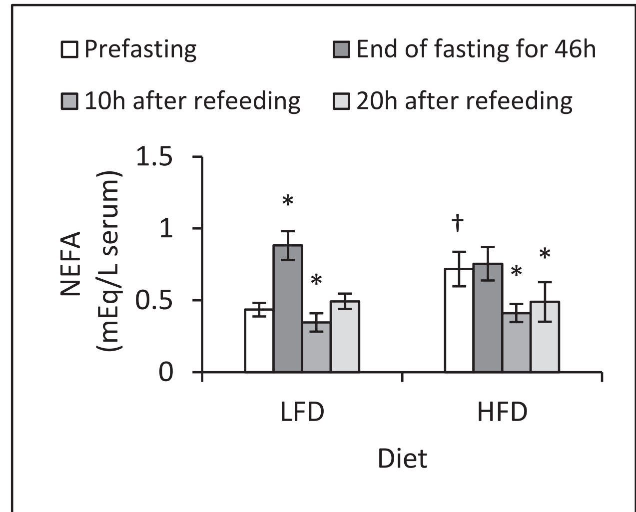

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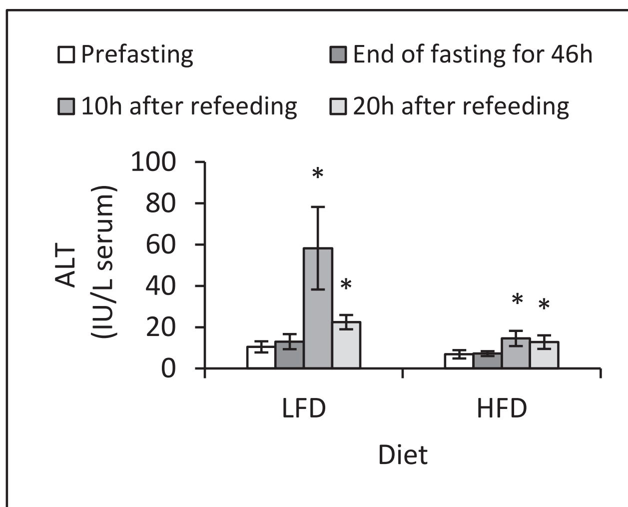

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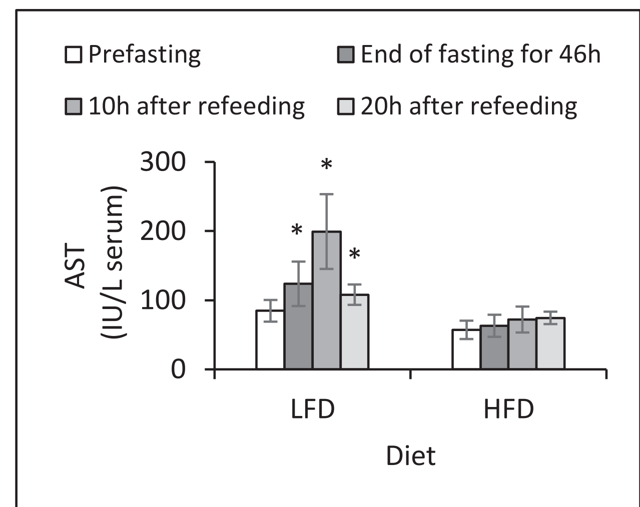
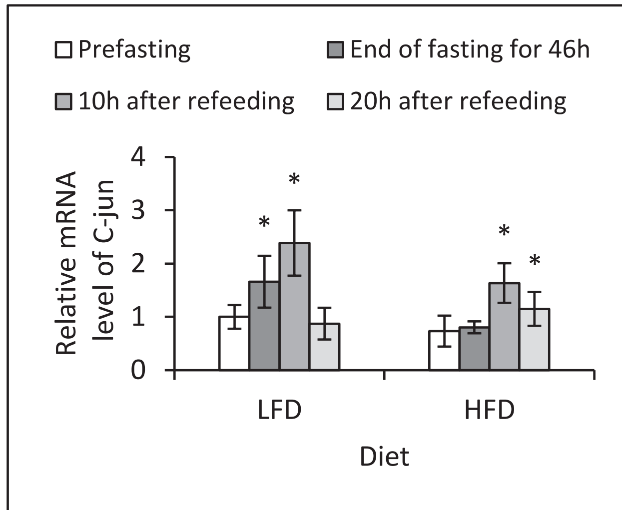


Fig.1. (A) Food intake and weights of (B) body, (C) liver, (D) abdominal white adipose tissue (WAT) and (E) brown adipose tissue (BAT), and serum levels of (F) glucose, (G) insulin, (H) nonesterified fatty acid (NEFA), (I) alanine aminotransferase (ALT) and (J) aspartate aminotransferase (AST) in mice fed a standard low-fat diet (LFD) or high-fat diet (HFD) for 94 days, fasted for 46h and then refed the same diets. Each value represents a mean ± standard deviation (n=7/test group). † Mean values were significantly different from the normal (prefasting) levels in the LFD-fed mice. *Mean values were significantly different from prefasting levels in each diet group.

HFD feeding for 94 days led to increased liver expression levels of cell markers for neutrophils (Mpo), eosinophils (Enpp3) and macrophages (TLR5) but had no significant effects on their expression levels of Emr1, another marker for macrophages (Prefasting, Fig.2C-F). The liver expression levels of neutrophil marker (Mpo) were maintained at prefasting levels in both diet groups until 20h after refeeding, but their expression levels of eosinophil marker (Enpp3) decreased below prefasting levels in the LFD and HFD groups at 10 and 0-20h after refeeding,

Fig.2

A



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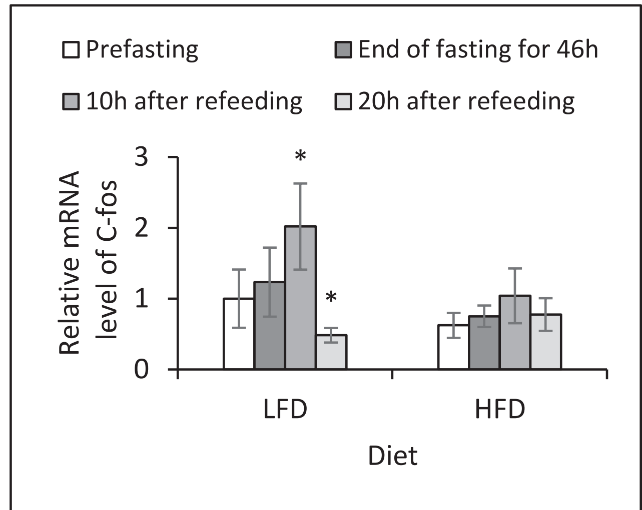

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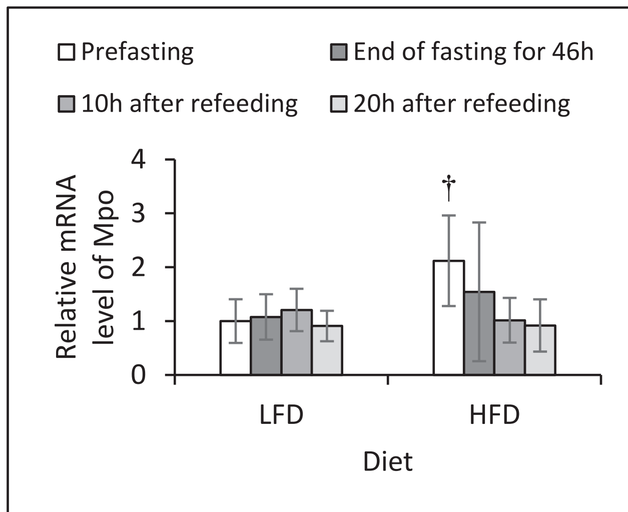

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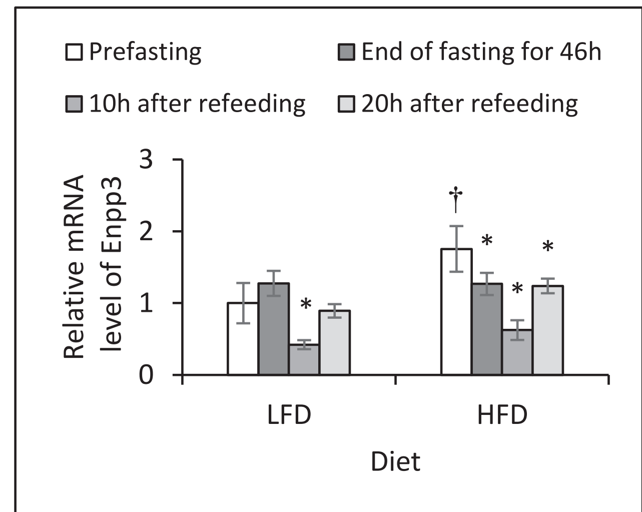

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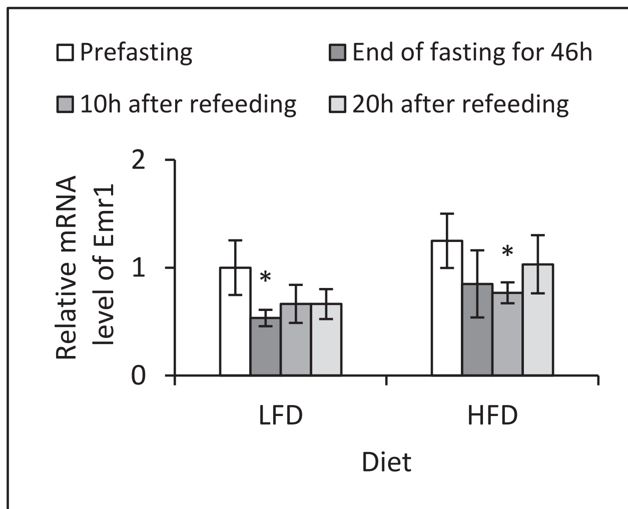

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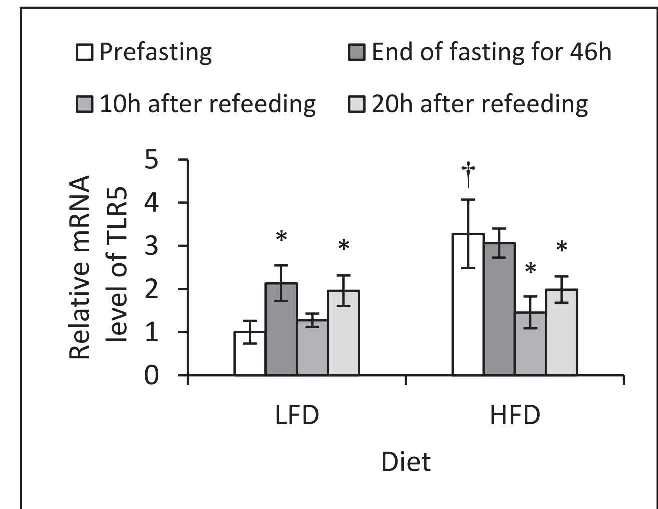

Fig.2

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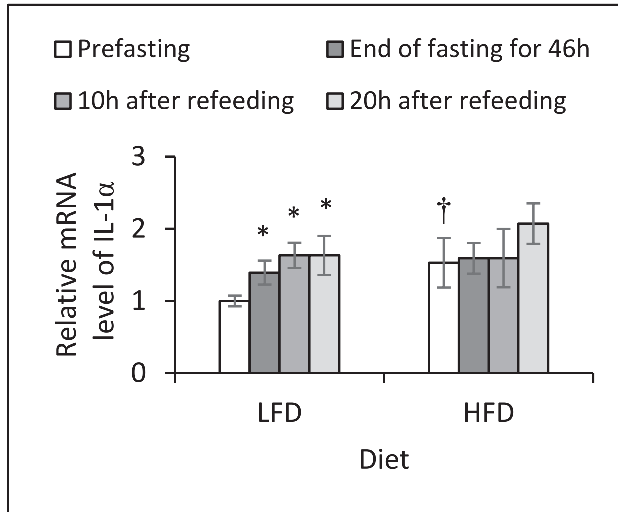

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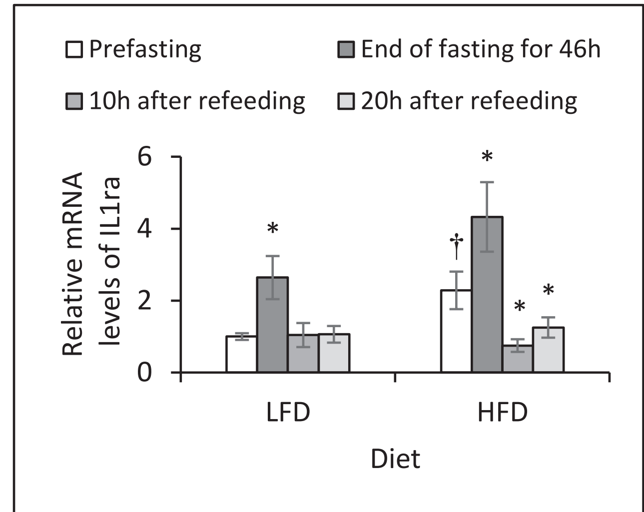


Fig.2. The liver mRNA levels for immediate-early genes (A, C-jun; B, C-fos), cell markers of neutrophils (C, Mpo), eosinophils (D, Enpp3) and macrophages (E, Emr1; F, TLR5), and pro- and anti-inflammatory cytokines (G, IL-1α; H, IL1ra) in mice fed a standard low-fat diet (LFD) or high-fat diet (HFD) for 94 days, fasted for 46h and then refed the same diets. Each value represents a mean ± standard deviation (n=7/test group). †Mean values were significantly different from the normal (prefasting) levels in the LFD-fed mice. *Mean values were significantly different from prefasting levels in each diet group.

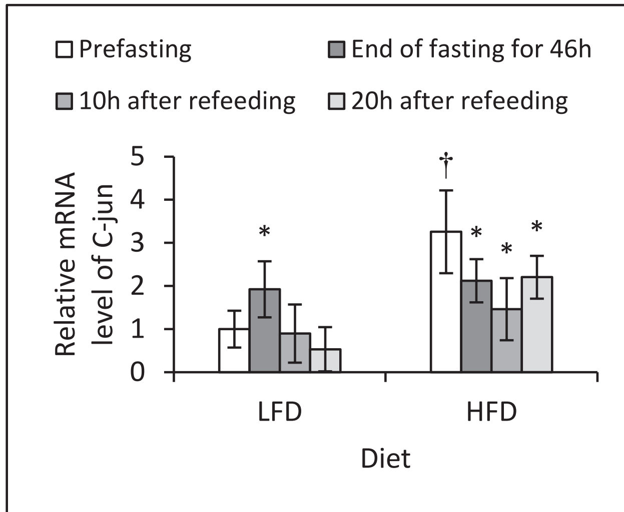
respectively. The liver expression levels of macrophage marker (Emr1) transiently decreased in the LFD and HFD groups at 0 and 10h after refeeding, respectively. The liver expression levels of another macrophage marker (TLR5) decreased below pre-fasting levels in the HFD-fed mice at 10-20h after refeeding. HFD feeding for 94 days resulted in elevated liver expression levels of pro-inflammatory cytokine (IL-1α) and interleukin receptor antagonist (IL1ra) compared with LFD feeding for the same period (Fig.2G and H). Their expression levels of pro-inflammatory cytokine (IL-1α) increased in the LFD-fed mice at 0-20h after refeeding but were maintained at prefasting levels in the HFD-fed mice until 20h after refeeding. Fasting for 46h resulted in a transient increase in the liver expression levels of interleukin receptor antagonist (IL-1ra) in both diet groups.

Expression of specific genes in the abdominal WAT

HFD feeding for 94 days caused an increase in abdominal WAT expression levels of immediate-early genes (C-jun and C-fos) compared with LFD feeding for the same period (Prefasting, Fig.3A and B). Their expression levels of C-jun transiently increased in the LFD group following fasting for 46h but decreased below pre-fasting levels in the HFD group at 0-20h after refeeding. Fasting and refeeding resulted in increased abdominal WAT expression levels of C-fos in both diet groups, which was greater in the LFD group. HFD feeding for 94 days had no significant effect on the abdominal WAT expression levels of neutrophil marker (Mpo) but resulted in elevated their expression levels of cell markers for eosinophils (Enpp3) and macrophages (Emr1, CD206) compared with LFD feeding (Prefasting, Fig.3C-F). Fasting for 46h led to a transient increase in abdominal WAT expression levels of

Fig.3

A



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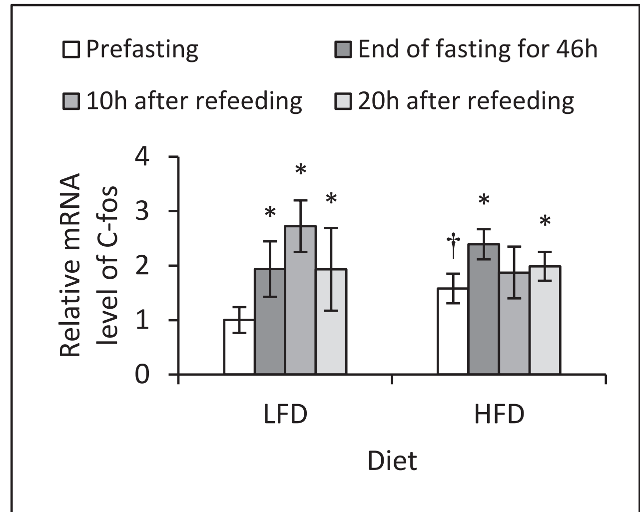

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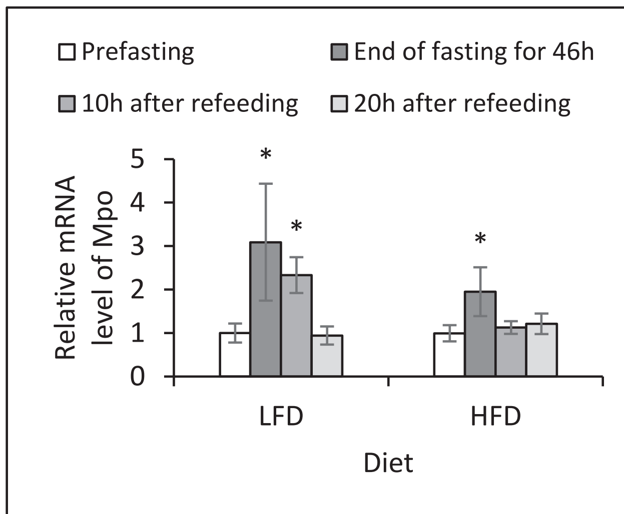

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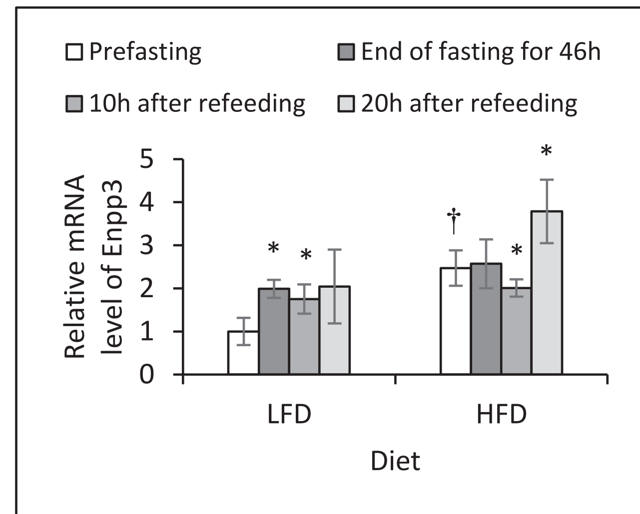

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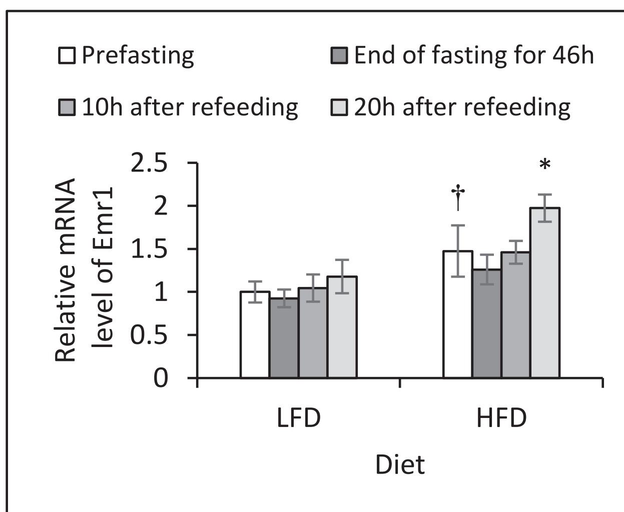

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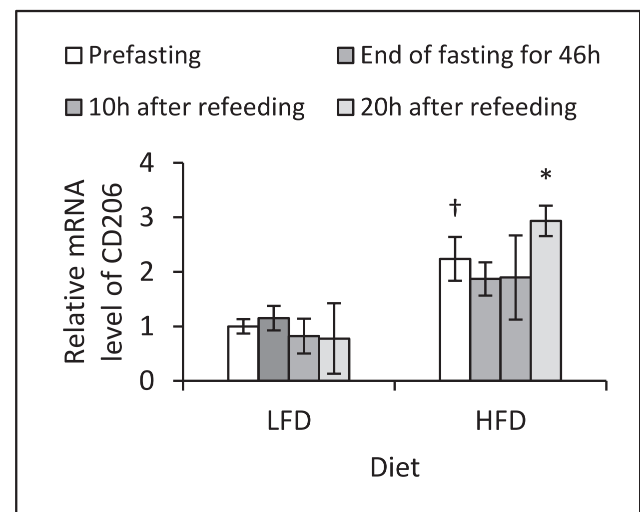

Fig.3

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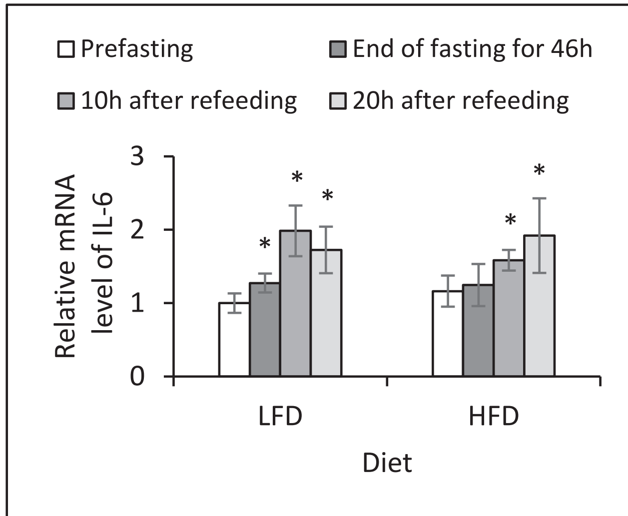

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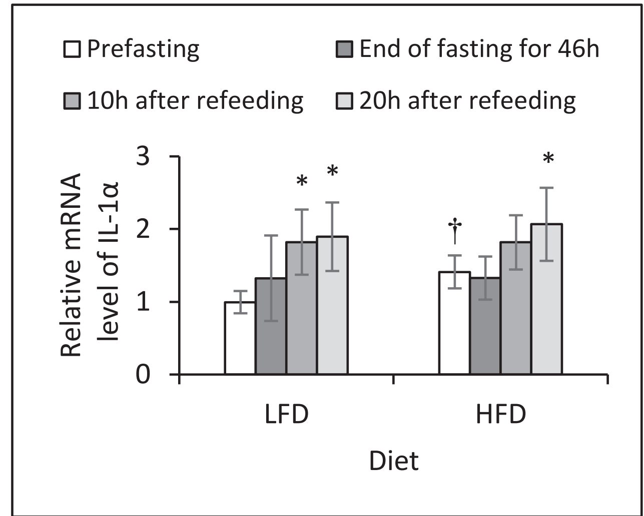

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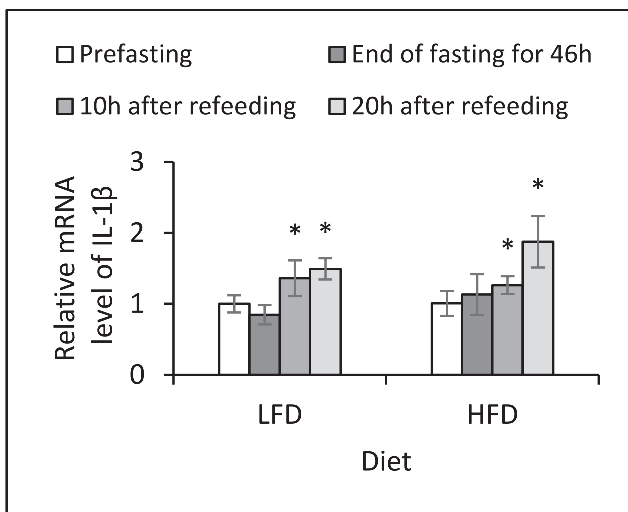

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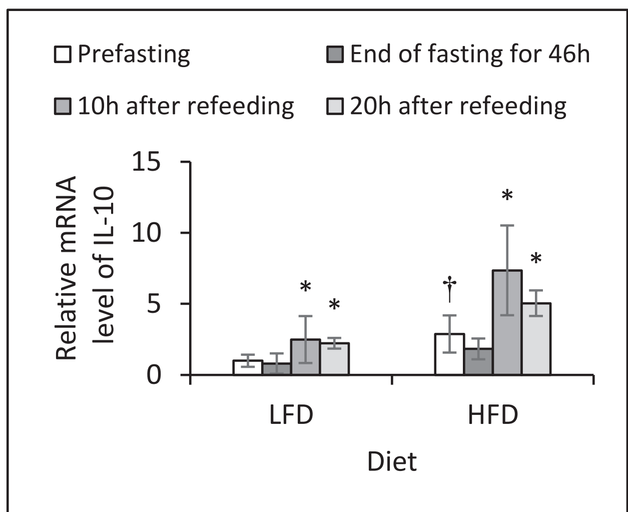

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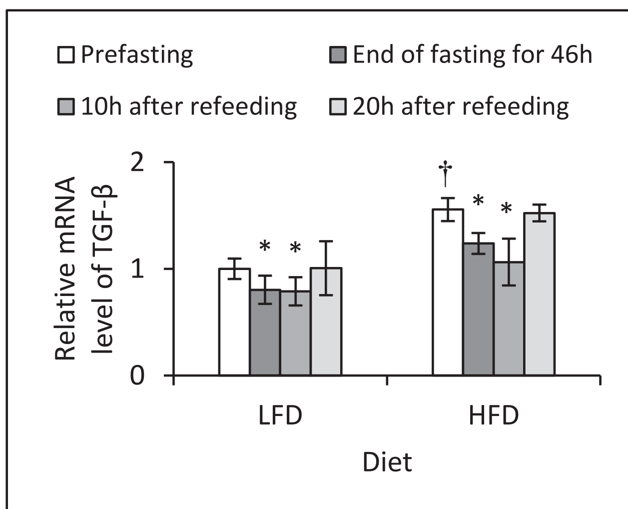


Fig.3. The abdominal white adipose tissue (WAT) mRNA levels for immediate-early genes (A, C-jun; B, C-fos) , cell markers of neutrophils (C, Mpo), eosinophils (D, Enpp3) and macrophages (E, Emr1 F, CD206), and pro- and anti-inflammatory cytokines (G, IL-6; H, IL-1α; I, IL-1β; J, IL-10, K, TGF-β) in mice fed a standard low-fat diet (LFD) or high-fat diet (HFD) for 94 days, fasted for 46h and then refed the same diets. Each value represents a mean ± standard deviation (n=7/test group). † Mean values were significantly different from the normal (prefasting) levels in the LFD-fed mice. *Mean values were significantly different from prefasting levels in each diet group.

neutrophil marker (Mpo) in both diet groups. Their expression levels of eosinophil marker (Enpp3) also increased in the LFD and HFD groups at 0-10 and 20h after refeeding, respectively. Abdominal WAT expression levels of macrophage markers (Emr1, CD206) were maintained at prefasting levels in the LFD group until 20h after refeeding but increased over prefasting levels in the HFD group at 20h after refeeding. HFD feeding for 94 days resulted in elevated abdominal WAT expression levels of several pro- and anti-inflammatory cytokines (IL-1α, IL-10, TGF-β) compared with LFD feeding (Prefasting, Fig.3H, J and K). In both diet groups, fasting and refeeding resulted in increased abdominal WAT expression levels of pro- and anti-inflammatory cytokines (IL-6, IL-1α, IL-1β and IL-10) (Fig. 3G and I). In contrast, their expression levels of TGF-β decreased in both diet groups following fasting and refeeding.

Expression of specific genes in the BAT

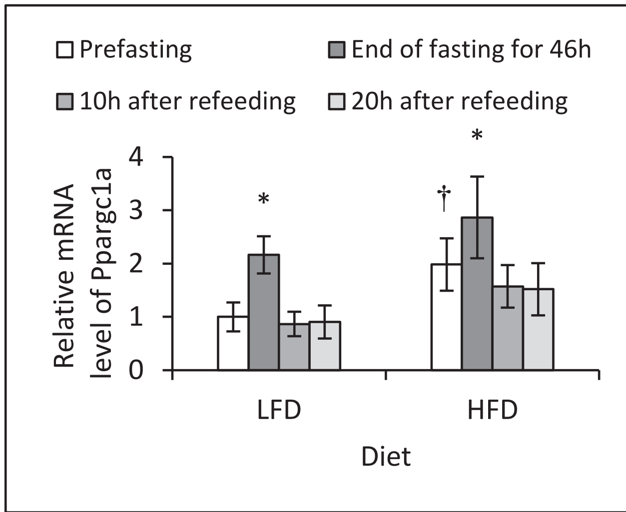
Ppargc1a and Acsl1 are involved in energy metabolism. Ppargc1a is a transcriptional coactivator that has been implicated in a wide array of human diseases including type II diabetes and other diet-related diseases[13]. Acsl1 is a major acyl-CoA synthetase of adipocytes[14]. HFD feeding for 94 days resulted in increased BAT expression levels of Ppargc1a and Acsl1 compared with LFD feeding for the same period (Prefasting, Fig.4A and B). Fasting for 46h caused transiently increased BAT expression levels of Ppargc1a in both diet groups, and led to transiently elevated BAT expression levels of Acsl1 in the LFD group. HFD feeding for 94 days resulted in decreased BAT expression levels of neutrophil marker (Mpo) and increased their expression levels of eosinophil marker (Enpp3) compared with LFD feeding (Prefasting, Fig.4C and D). Fasting for 46h resulted in transiently increased BAT expression levels of neutrophil marker (Mpo) and decreased thier expression levels of eosinophil marker (Enpp3) in both diet groups. HFD feeding for 94 days did not significantly affect BAT expression levels of macrophage cell surface marker (Emr1), but led to decreased their expression levels of intracellular macrophage marker (COX2) (Fig.4E and F). BAT expression levels of Emr1 slightly decreased in the LFD group but were maintained at prefasting levels in the HFD group until 20h after refeeding. BAT expression levels of COX2 increased over prefasting levels in both diet groups at 10-20h after refeeding. HFD feeding for 94 days had no significant effects on BAT expression levels of pro-inflammatory cytokines (IL-6. IL-18) (Fig.4G and H). Their IL-6 and IL-18 expression levels decreased in the LFD-fed mice but increased over prefasting levels in the HFD-fed mice following fasting and refeeding.

Discussion

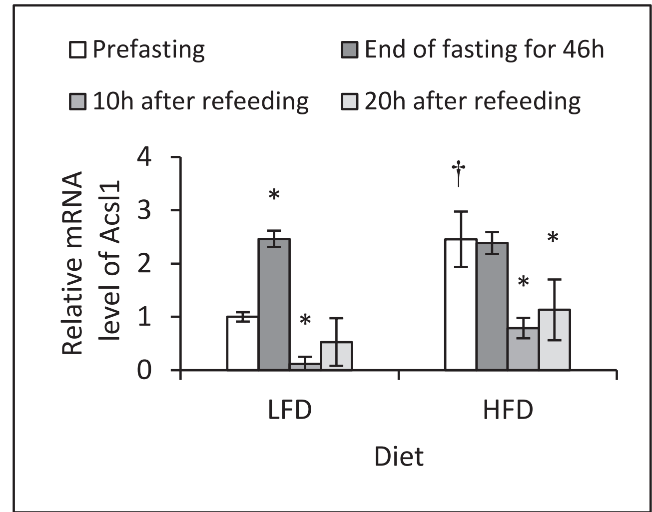
In the present study in mice that were fed a standard LFD for 94 days, refeeding after the 46h fast resulted in a rapid and abnormal increase in serum ALT and AST levels as observed in a previous study[2,3], and led to increased liver expression levels of immediate-early genes (C-jun, C-fos). These effects were, however, significantly attenuated in mice fed HFD for 94 days. It is noteworthy in this regard that the expression of immediate-early genes such as C-jun and C-fos has been shown previously to increase in the liver after various insults, and to be associated with severe hepatic damage, followed by liver regeneration[15]. Thus, the results of the present study indicate that HFD feeding may attenuate fasting and refeeding-induced hepatocellular injury. In the LFD-fed mice, liver weight decreased to 77% of normal prefasting levels following fasting for 46h and then rapidly recovered to 168% of the levels that is observed after fasting. In contrast, in the HFD-fed mice, the liver weight was maintained at prefasting levels until the end of fasting for 46h and then increased to 138% of the levels that is observed after fasting. These results indicate that fasting and refeeding-induced severe alterations in the liver weight is attenuated in the HFD-fed mice when compared with the LFD-fed mice. This attenuation may, at least in part, account for the reduced acute

Fig.4

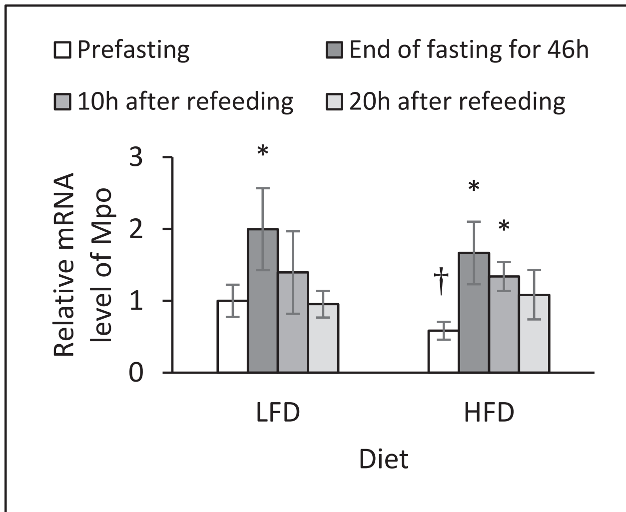
A



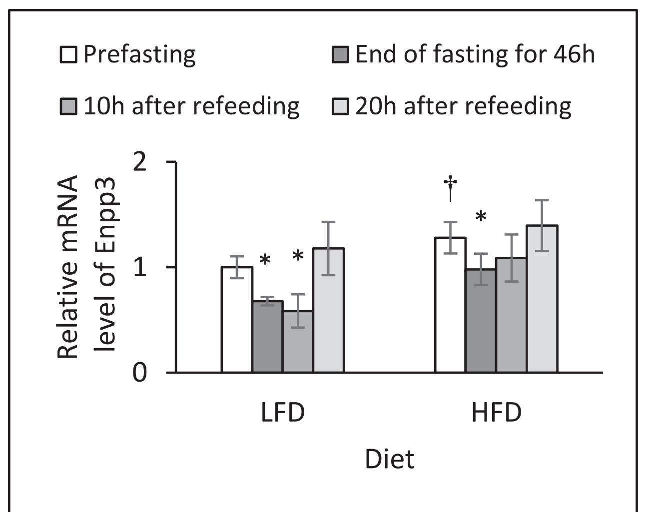
B



C



D



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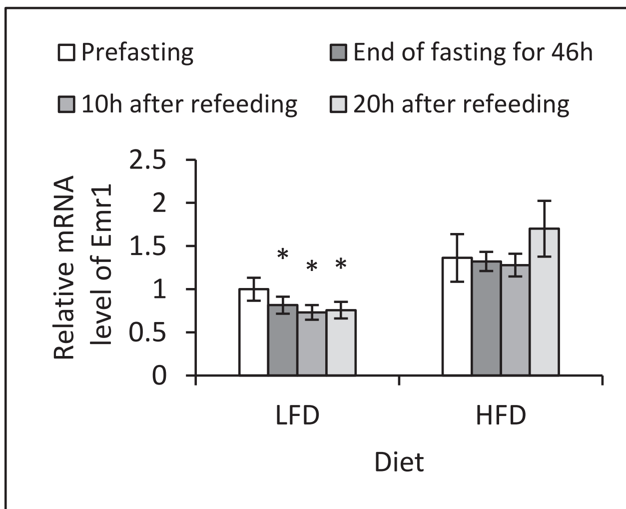

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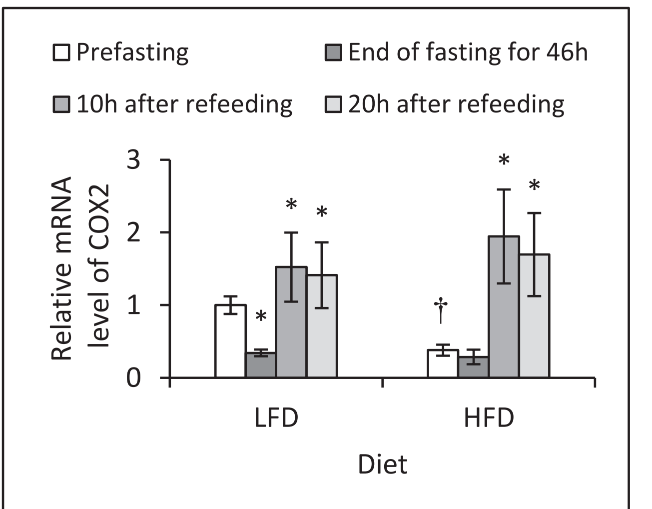
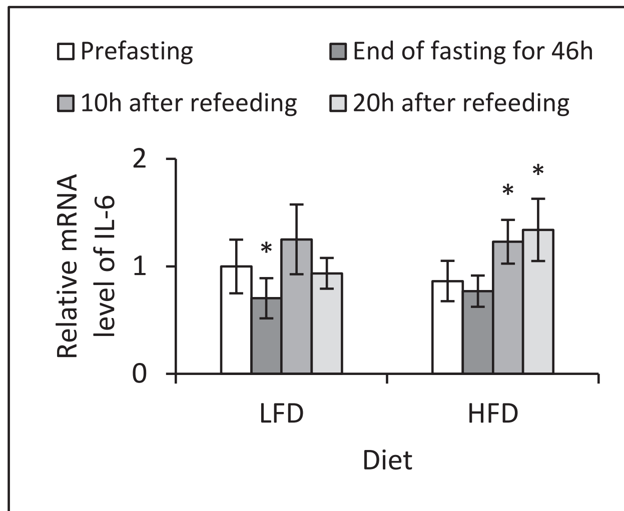


Fig.4

G



H

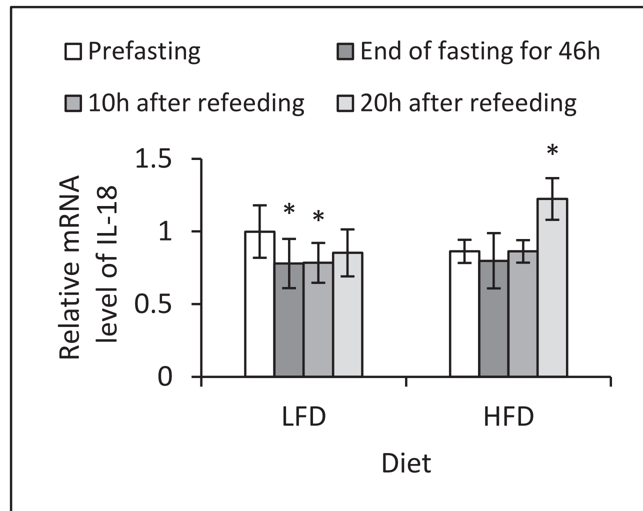


Fig.4. The brown adipose tissue (BAT) mRNA levels for genes involved in energy metabolism (A, Ppargc1a; B, Acsl1), cell surface markers of neutrophils (C, Mpo), eosinophils (D, Enpp3) and macrophages (E, Emr1 F, COX2) and pro-inflammatory cytokines (G, IL-6; H, IL-18) in mice fed a standard low-fat diet (LFD) or high-fat diet (HFD) for 94 days, fasted for 46h and then refed the same diets. Each value represents a mean ± standard deviation (n=7/test group). † Mean values were significantly different from the normal (prefasting) levels in the LFD-fed mice. *Mean values were significantly different from prefasting levels in each diet group.

hepatocellular injury in the HFD-fed mice.

It is well known that WAT is an important site of inflammatory events in HFD-induced obesity and that a key event in obesity is the macrophage infiltration of WAT[8, 9]. In the present study, mice fed HFD for 94 days exhibited higher abdominal WAT expression levels of macrophage markers (Emr1, CD206) and several pro- and anti-inflammatory cytokines (IL-1α, IL-10, TGF-β) when compared with mice fed standard LFD for the same period. In addition, the HFD-fed mice showed fasting and refeeding-induced increase in their expression levels of macrophage markers (Emr1, CD206) and pro- and anti-inflammatory cytokines (IL-6, IL-1α, IL-1β and IL-10). These findings suggest that HFD enhances abdominal WAT inflammatory response, and this enhancement is augmented by a fasting-refeeding regime. IL-10 has a central role in infection by limiting the immune response to pathogens and thereby preventing damage to the host. In our present analysis, HFD feeding for 94 days resulted in increased abdominal WAT expression levels of IL-10 and TGF-β compared with LFD feeding, and refeeding after the 46h fasting led to further increase in their expression levels of IL-10 but not of TGF-β. We therefore speculate that IL-10 may contribute to prevent excessive inflammation induced by a fasting-refeeding regime under HFD feeding.

Ppargc1a plays an important role in maintaining blood glucose levels by up-regulating genes involved in gluconeogenesis and the beta-oxidation of fatty acids[16]. In our present study, fasting for 46h resulted in a transient increase in BAT expression levels of ppargc1a in both diet groups. This increase may be attributed to the 46h fasting-induced marked decrease in serum glucose levels. The long-chain acyl-coenzyme A synthetase (ASCL) family plays a key role in

regulating fatty acids which enter the oxidative or synthetic pathways to generate energy or produce phospholipids, cholesterol esters and triglycerides[17,18]. We anticipated that fasting-induced increased BAT expression levels of Acsl1 in the LFD-fed mice were due to a severe lack of energy and may contribute to promote fatty acid β-oxidation. Refeeding resulted in increased BAT expression levels of pro-inflammatory cytokines (IL-6, IL-18) in the HFD-fed mice, but not in the LFD-fed mice. These results suggest that HFD feeding may promote refeeding-induced BAT inflammatory response.

In summary, our present results indicate that a fasting-refeeding regime in combination with HFD feeding for a long period (94 days) causes an attenuation of hepatocellular injury but promotes inflammatory response in abdominal WAT and BAT compared with a fasting-refeeding regime alone. A state of low-great and chronic inflammation can play an important role in the development of obesity-associated metabolic diseases. The combined effects of HFD and repeat fasting-refeeding in the inflammatory state of abdominal WAT and BAT warrant further study to elucidate the involvement of irregular eating behaviors and HFD in the pathogenesis of these metabolic diseases.

Abbreviation

Acsl1, acyl-CoA synthetase 1; CD206, CD206 antigen; COX2, cytochrome c oxidase II; Emr1, adhesion G protein-coupled receptor E1; Enpp3, pyrophosphatase/phosphodiesterase 3; IL, interleukin; Il1ra, interleukin 1 receptor antagonist; Mpo, myeloperoxidase; Ppargc1a, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TGF-β, transforming growth factor-beta; Tlr5, toll-like receptor 5.

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