

Effect of fasting and refeeding on the consequences of myocardial infarction in rats

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Abstract

We investigated the effects of 48 hours (h)-fasting followed by permanent coronary artery occlusion and 48 h-refeeding on the infarct size; ejection fraction; plasma creatine kinase activity; and levels of IL1- β , CINC 2 α/β and IL-6, triglycerides, glycogen and ATP in the left ventricle of adult rats. Permanent coronary artery occlusion was performed in ad libitum fed (fed/fed) and 2-days-fasted rats (fasted/re-fed). After infarction induction, both groups were re-fed for 48 h, and infarction area and ejection fraction were assessed using echocardiography. The levels of glycogen, triglyceride, ATP, and proinflammatory cytokines in the left ventricle, and the concentrations of glucose and free fatty acids in plasma were measured at the end of the 2-day fasting period. The infarct size was significantly smaller in the fasted/re-fed group compared with the fed animals. This was accompanied by decreased plasma creatine kinase activity and improved ejection fraction. Fasting per se led to an increase in glycogen, ATP, CINC 2 α/β , interleukin (IL)-1 β , and IL-6, and a decrease in triacylglycerol levels in the left ventricle; along with an increase in free fatty acid concentrations and a decrease in glucose levels in the plasma. This study indicates that 2-days-fasting followed by 48 h refeeding protected the heart from ischemia-induced injury, which may be related to the fasting-induced metabolic condition.

Introduction

Acute myocardial infarction (AMI) is a major cause of mortality and morbidity worldwide [1]. AMI is initiated by a lack of blood flow resulting in marked reduction in the supply of oxygen and nutrients to the cardiac muscle. This in turn leads to a decrease in ATP production and causes oxidative stress, loss of ion gradient across membrane, accumulation of toxic metabolites, and inflammation [2]. Fatty acid (FA) oxidation is the main source of ATP to the heart in aerobic conditions [3,4]; however, during ischemia, ATP production occurs through anaerobic glycolysis using glucose derived from glycogen [4].

Expression of pro-inflammatory cytokines, such as IL-1 β , tumor necrosis factor (TNF)- α , and IL-6, usually absent in the normal heart, is increased in the hearts of animals and humans affected by AMI [5,6]. The influx of inflammatory cells and production of pro-inflammatory mediators contribute to myocardial injury. However, broad inhibition of inflammation has adverse effects on outcomes following an AMI [7,8]. Inflammation is not an injurious process *per se*, since it triggers the first steps for proper AMI healing [9,10].

Dietary restriction increases cardiac tolerance to ischemic injury [11,12]. Intermittent fasting increases capillary density [13], reduces the number of apoptotic cells, and decreases the inflammatory response in the ischemic heart [14]. A fasting period of one to three days protects from renal [15] and hepatic [16] ischemic/reperfusion injury in mice.

Nevertheless, the impact of short-term fasting followed by short-term re-feeding on cardiac tolerance to ischemic injury, dissociated from reperfusion damage, has not been examined yet. In the present study, the infarct size, ejection fraction (EF), left ventricle (LV) mass, and levels of ATP, triglycerides (TG), glycogen and proinflammatory

cytokines in the LV of rats subjected to fasting for 2 days followed by permanent coronary artery occlusion and 48 h re-feeding were investigated.

Materials and methods

Animals and the experimental protocol

Male Wistar rats weighing 260-280 g were obtained from the Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo. Animals were housed under a 12:12 h light-dark cycle at 23 \pm 1°C.

The rats were randomly assigned to two experimental groups: fed *ad libitum* (fed/fed) or 48 h fasting and 48 h refeeding (fasted/re-fed). Six animals were used from the fed and fasted groups to evaluate the metabolic/inflammatory conditions before infarction. Animals in the fasted/re-fed group did not have access to food for 48 h prior to infarct induction. The animals were fed a standard laboratory chow (NUVILAB CR-1, Nuvital, Curitiba, Brazil). All groups received water *ad libitum*. The ethical committee of the Institute of Biomedical

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Sciences of the University of São Paulo approved this study.

Induction of AMI

Surgical occlusion of the left coronary artery was performed between 5:00 pm and 7:00 pm as previously described [17-20]. Briefly, left thoracotomy was performed, the third intercostal space dissected, and the heart exposed. The left coronary artery was occluded with a single nylon (6.0) suture 1 mm distal to the left atrial appendage. The chest was closed using silk sutures. The animals were maintained under ventilation until recovery. All groups received standard laboratory chow and water *ad libitum* after surgery.

Assessments of ejection fraction and infarct size

Echocardiography was performed 48 h after coronary ligation, according to the guidelines of the American Society of Echocardiography. Rats were anesthetized (80 mg ketamine and 12 mg xylazine per kg b.w.), and images were obtained with a 10–14 MHz linear transducer in a SEQUOIA 512 Echocardiographic System (ACUSON Corporation, Mountain View, CA, USA) for the measurement of infarct size and systolic function (ejection fraction). MI size was estimated on the basis of identification of akinetic (absence of movement), hypokinetic (decreased movement), or dyskinetic (paradoxical movement) areas [17,18,20]. MI size (% of LV perimeter) was measured as the ratio of dysfunctional area to the total perimeter of the endocardial border of the LV.

Cytokine levels in the LV

ELISA Duo-set kits were used for the determination of IL-1 β , CINC-2 α/β , and IL-6 in LV protein extracts (R&D Systems Inc., Minneapolis, MN, USA). The results were normalized by total protein levels.

Measurements of ATP

Left ventricles were dissected and stored in liquid nitrogen prior to ATP assay. The tissues were homogenized in a Polytron PT 3100 at position 3/4 (Littau-Luceme, Switzerland). Perchloric acid (0.6 N) was used as the extraction medium and the supernatants were neutralized using 1 M potassium bicarbonate. ATP concentrations were determined in the supernatants using an ATP determination kit (Invitrogen, Eugene, Oregon, USA). ATP contents were calculated as μM and expressed with respect to tissue wet weight.

Glycogen content in the LV

Glycogen content of the LV was determined using the method of Keppler and Decker [21]. This method is based on the conversion of glycogen into glucose by amyloglucosidase (Sigma, St. Louis, MO, USA). Glucose from glycogen was quantified using a glucose determination kit (Bioclin, Belo Horizonte, MG, Brazil). Glycogen content was calculated as mg of glycosyl units per gram wet weight of the LV.

Triglyceride content in the LV

For measurement of TG content in the LV, 60 mg of frozen heart tissue was homogenized in 2:1 chloroform and methanol solution. The homogenate was centrifuged at 1,000 g for 20 min at 4°C. The lower phase was transferred to a new tube and evaporated in a vacuum centrifuge (Vacufuge® plus Eppendorf). The lipid pellets were suspended in 100 μL absolute ethanol. The TG content was measured by spectrophotometry using a commercial kit (Bioclin, Belo Horizonte, MG, Brazil).

Glucose and free fatty acid (FFA) levels and CK activity

Blood samples of fed and fasted/re-fed rats were collected into tubes containing EDTA through cardiac puncture. Fed rats were subjected to 6 h fasting before blood samples being collected. The blood samples were centrifuged at 2,000 rpm for 15 min at 4°C, and the serum was separated and frozen. Plasma CK activity and glucose concentration were determined using commercial kits (Bioclin, Belo Horizonte, MG, Brazil). Plasma FFA levels were estimated as reported by Falholt *et al.* [22].

Statistical analysis

Results are presented as mean \pm SEM. Student's *t* test was used for comparison between the two groups: fed/fed vs. fasted/re-fed and fed vs. fasted. Differences between values were considered statistically significant at $p < 0.05$. All results were analyzed using the GraphPad Prism 5.0 statistical software (GraphPad Software, San Diego, CA, USA).

Results

The body weight of the fed/fed group remained unchanged during the 96 h of experiment. However, the body weight of fasted/re-fed animals was decreased (by 19%) after 48 h fasting but remained unchanged afterwards (Figure 1).

Before myocardial infarction

Plasma glucose levels in the fasted group were 37% lower, whereas plasma FFA concentrations were increased 2-fold due to fasting for 48 h (Table 1). The ATP concentration was 33% higher, whereas the glycogen content was 4-fold higher in the LV of the fasted compared with the fed group (Figures 2A-2B). The TG content in the LV of hearts from fasted rats was 61% lower compared with the fed group (Figure 2C).

The levels of CINC-2 α/β , IL-1 β , and IL-6 were higher (by 3.2-, 4.9-, and 1.4-fold, respectively) in the fasting compared with the fed group (Figures 3A-3C).

After myocardial infarction

The size of the infarct, measured using echocardiography 48 h after ischemia induction, was 37% smaller in fasted/re-fed rats compared with the fed/fed group (Figure 4A). In addition, the ejection fraction was 31% higher in the fasted/re-fed compared with the fed/fed group (Figure 4B). Plasma CK activity was lower in the fasted/re-fed compared with the fed/fed group (Figure 5A). Similarly, the LV/heart weight ratio was decreased in the fed/fed compared with the fasted/re-fed animals (Figure 5B). The levels of CINC-2 α/β , IL-1 β , and IL-6 in the LV (Figures 6A-6C) and the plasma FFA levels (Figure 7) were not different among the groups.

Discussion

The key finding of this study was that fasting for 48 h prior to the induction of infarction followed by refeeding limited the extent of the infarcted area and prevented the expected systolic dysfunction

	Fed	Fasted
Glucose (mg/dL)	113.6 \pm 3.26	71.40 \pm 3.61**
Free fatty acids (mM)	0.32 \pm 0.017	0.62 \pm 0.072**

Values are expressed as mean \pm SEM. ** $P < 0.01$ vs the fed group.

Table 1. Plasma glucose and free fatty acid concentrations in the *ad libitum* fed (fed) and 48 h fasted (fasted) groups before infarct induction.

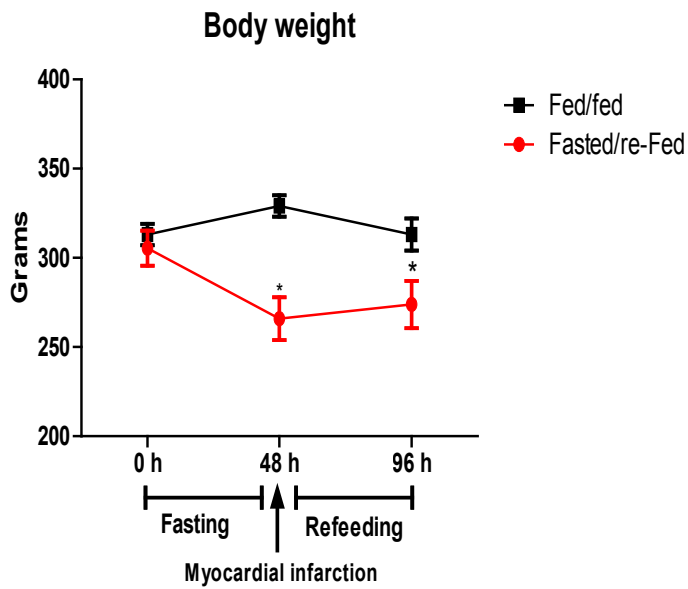


Figure 1. Body weight (g) before (48 h) and after (96 h) infarct induction in fed/fed and fasted/re-fed rats. Values are expressed as mean \pm SEM of six rats per group. (* $P < 0.05$) compared with the zero hour period.

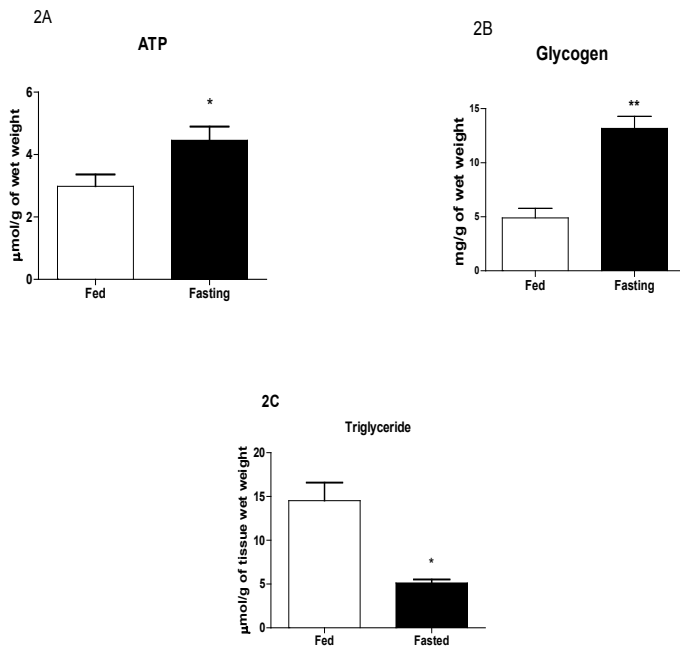


Figure 2. Contents of ATP (A), glycogen (B), and TG (C) in the left ventricle before infarct induction. The following groups were studied: Fed *ad libitum* (Fed) and 48 h fasted (Fasted). Values are expressed as mean \pm SEM of six animals per group. * $P < 0.05$; ** $P < 0.01$ compared with the Fed group.

partially. Additionally, fasting for 48 h *per se* led to an increase in the levels of ATP, glycogen, CINC-2 α/β , IL-1 β , and IL-6, and a reduction in the TG levels, in the LV.

Consistent with our observations, Snorek *et al.* [23] demonstrated that three-day fasting effectively reduces infarct size and ventricular arrhythmias under ischemia/reperfusion. Within 24 h after coronary ligation, death by necrosis is the primary cause of cardiomyocyte loss [24]. The reduced infarct size observed here reflects the protective effect of fasting on necrosis, as suggested by the lower plasma CK activity and

preservation of the LV mass after infarction.

The mechanism mediating the cardioprotection induced by fasting/refeeding remains to be fully elucidated. However, the increase in glycogen content in the heart before infarction possibly plays an important role. In the isolated heart experimental model, glycogen

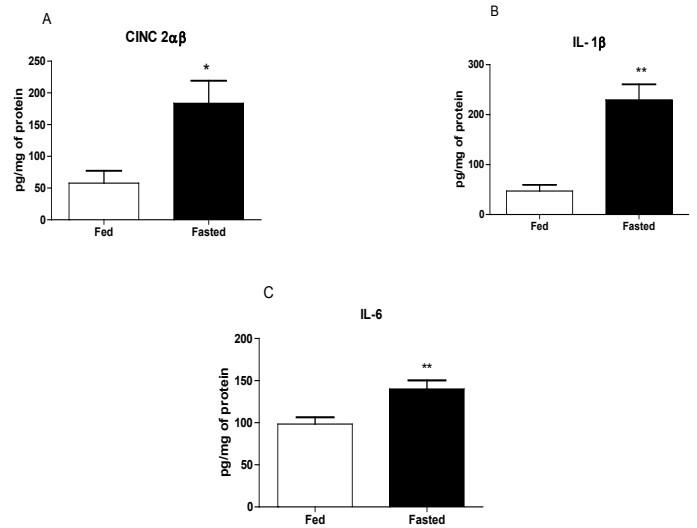


Figure 3. Levels of CINC-2 α/β , IL-1 β , and IL-6 in the left ventricle before infarction. The following groups were studied: Fed *ad libitum* (Fed) and 48 h fasted (Fasted). Values are expressed as mean \pm SEM of six animals per group. * $P < 0.05$; ** $P < 0.01$ compared with the Fed group.

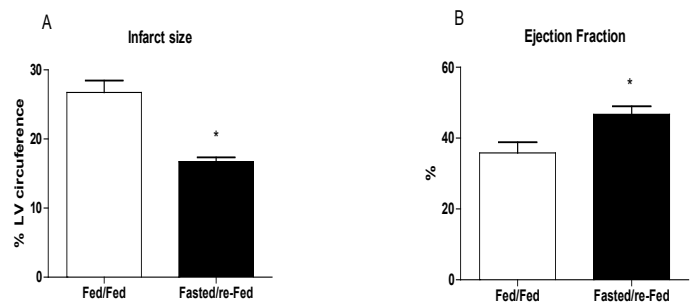


Figure 4. Infarct size and ejection fraction 48 h after infarct induction. The following groups were studied: Fed *ad libitum* (Fed/Fed) and 48 h fasting/re-feeding (Fasted/re-Fed). Values are expressed as mean \pm SEM of at least seven animals per group. * $P < 0.05$ compared with the Fed/Fed group.

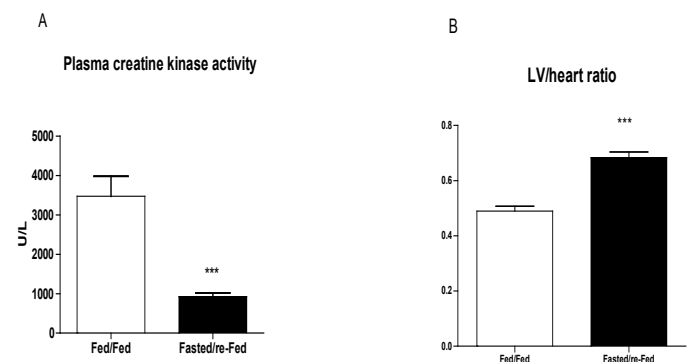


Figure 5. Creatine kinase activity in plasma and LV/heart weight ratio 48 h after infarct induction. The following groups were studied: Fed *ad libitum* (Fed/Fed) and 48 h fasting/re-feeding (Fasted/re-Fed). Values are expressed as mean \pm SEM of at least six animals per group. *** $P < 0.001$ compared with the Fed/Fed group.

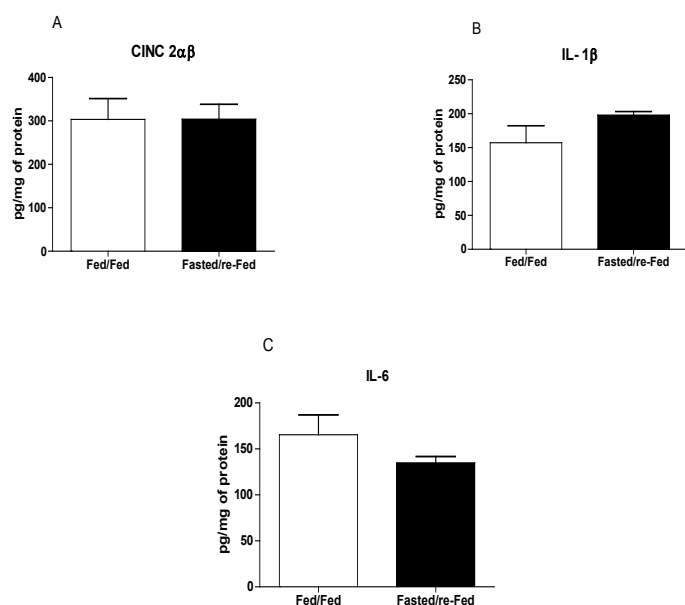


Figure 6. Levels of CINC-2αβ, IL-1β, and IL-6 in the left ventricle 48 h after infarct induction. The following groups were studied: Fed *ad libitum* (Fed/Fed) and 48 h fasted/re-fed. Values are expressed as mean ± SEM of six animals per group.

Plasma Free Fatty acid

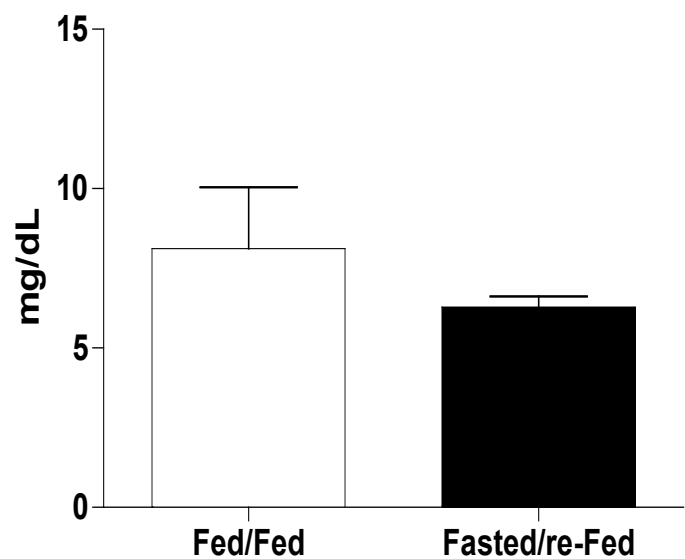


Figure 7. Free fatty acid concentrations in plasma 48 h after infarct induction. The following groups were studied: Fed *ad libitum* (Fed/Fed) and 48 h Fasted/re-Fed. Values are expressed as mean ± SEM of seven animals per group.

accumulation, induced by fasting or insulin administration, has been shown to increase ATP and ADP levels, normalize glucose utilization, reduce cardiomyocyte membrane damage, and shorten recovery time after ischemia [25]. Here, we showed that fasting for 48 h followed by permanent coronary artery occlusion and 48 h re-feeding is an effective protocol to protect the heart from ischemic injury.

Kokubun *et al.* [26] showed that fasting for 12 h and 48 h leads to an increase in the glycogen content of the heart, while reducing the

glycogen content in the skeletal muscle and liver in rats. Randle *et al.* [27] postulated that increased plasma FFA concentrations reduce glucose utilization and lead to glycogen accumulation in the heart. Suzuki *et al.* [28] reported that serum FFA concentrations increase two-fold in 16 h- and 48 h-fasted mice. A two-fold increase in plasma FFA concentrations was also observed in our study after fasting for 48 h. FFAs are used preferentially as fuels in aerobiosis and account for 60-90% of the ATP produced in the heart under non-ischemic conditions [3,4]. In the present study, the decrease in TG levels in the LV of animals fasted for 48 h point to an increase in FA oxidation before infarction induction. This was associated with an increase in ATP content. However, under anaerobic conditions, FA oxidation is decreased and ATP production is dependent on anaerobic utilization of glucose derived from glycogen degradation [4,26].

Cell survival following acute myocardial ischemia requires marked changes in metabolism in the heart. Goodwin *et al.* [29] showed that under adrenergic stimulation, glucose uptake by the perfused heart is delayed in relation to the burst of glycogenolysis, indicating the important role of glycogen in stress conditions. Therefore, increased levels of FFAs in the plasma, and of ATP and glycogen in the LV after 48 h-fasting are part of the adaptive metabolic changes that protect the heart from an infarct-induced injury.

Liepinsh *et al.* [30] reported an increase in the infarct size in *ex vivo* perfused heart from fasted compared with fed rats. This effect was observed immediately post-reperfusion in the ischemia/reperfusion model. They used a Krebs-Henseleit buffer solution supplemented with metabolites for reperfusion to mimic the blood plasma parameters found in the fasted state. In the fasted state, plasma concentrations of FFA are elevated [28]. FA oxidation overrides glucose oxidation, which decreases the contribution of anaerobic glycolysis to energy production in the myocardium. The differences between our study and that of Liepinsh *et al.* may be explained by the fact that we measured the infarcted area after 48 h of refeeding, when the plasma fatty acid concentrations were not elevated. Hence, the elevated plasma FFA levels in the fasting state are necessary for metabolic changes (increase in glycogen and ATP in LV), but may be detrimental in MI condition; impairing glucose metabolism if permanently elevated. Malfitano *et al.* [20] demonstrated cardioprotection from ischemia injury in streptozotocin-induced diabetic rats after two weeks of tissue remodeling. Like in the fasting, they showed that the diabetic condition *per se* increased glycogen in the LV and FFA plasma concentration before infarct induction and, under low plasma and tissue FFA concentrations, systolic dysfunction and infarct size were smaller when compared with non-diabetic animals. Furthermore, previous studies, using *ex vivo* isolated perfused hearts, concluded that hearts from fasted rats are better protected from injury induced by ischemia/reperfusion [26,31,32]; these results are in agreement with our findings. These studies used buffer solution for perfusion that closely mimics the fed state with low plasma FFA levels.

We found increased amounts of CINC-2αβ, IL-1β, and IL-6 in the LV from the fasted group before MI induction. Verweij *et al.* [16] reported that the increased mRNA expression of IL-6 and P-selectin played a protective role against ischemia/reperfusion injury in the liver of 3-day-fasted mice.

Excess plasma FFAs can be harmful to cardiac tissue [33]. Zhou *et al.* [34] demonstrated that cardiac dysfunction in obesity is caused by apoptosis of cardiomyocytes due to exposure to high FFA levels. Under fed conditions, FFAs are mainly derived from the hydrolysis of

TG-rich lipoproteins on the endothelial surface of coronary arteries. During fasting, reduced glucose utilization triggers FA release from the heart TG, leading to FA supply far beyond the oxidation capacity of the tissue. This excess of FFAs causes cell death that has been defined as “cardiac lipotoxicity” [35]. This is likely the mechanism by which fasting for 48 h causes inflammation in the LV. In the fasting state, cardiomyocytes are exposed to high plasma FFA concentrations and to FFAs from the heart TG stores.

The metabolic changes (glycogen and ATP accumulation and intense TG mobilization) induced by 48 h fasting put the heart in an “alert” state, as indicated by increased CINC-2 α/β , IL-1 β , and IL-6 levels in the LV, to deal with eventual injury episodes. The smaller infarct size and normal ejection fraction observed in the fasted/re-fed group may be a consequence of the metabolic adaptation imposed by fasting.

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Author contributions

A.L.S.J., M.C.I., and R.C. designed the experiments. A.L.S.J., C.M., D.F., and L.E.S. performed the experiments. A.L.S.J., M.C.I., and RC analyzed and interpreted the data. The article was revised by M.C.I. and R.C. A.L.S.J. and E.I. wrote the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest

The author(s) declare that they have no conflicts of interest.

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