Effects of caloric restriction on oxidative stress parameters

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Abstract. Moderate caloric restriction prolongs lifespan. Changes in oxidative stress and hormesis may be involved in this process. The aim of this study is to examine the effects of different levels of chronic caloric restriction (CR) and acute fasting on stress response and oxidative stress parameters in rat liver and plasma. Forty-two rats were divided into groups: control group, calorie-restricted groups with intake of 80–90%, 60–70%, 40–50%, 20–30% of daily caloric needs and acute fasting group. To determine alanine aminotransferase (ALT), aspartate aminotransferase (AST) and superoxide dismutase (SOD) activity, concentration of corticosterone, nitrites and nitrates (NO_x), malondialdehyde (MDA) and glutathione (GSH), liver samples and blood were collected. Increase in plasma corticosterone concentration and AST and ALT activity was found in severe CR. Ingestion 40–50% daily caloric needs or less increased liver MDA and NO_x concentration and decreased SOD activity. Ingestion 60–70% daily caloric needs increased Mn-SOD activity, GSH and NO_x. In acute fasting group and group taking 20–30% daily caloric needs, GSH was significantly lower than in control group. Severe CR and acute fasting increase oxidative damage and decrease antioxidative capacity of hepatocytes. Moderate CR increases antioxidative capacity of hepatocytes due to increase in Mn-SOD activity and GSH concentration, which might have a role in anti-aging and hormetic mechanism of CR.

Key words: Caloric restriction — Oxidative stress — Hormesis — Acute fasting

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CR, caloric restriction; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); eNOS, endothelial nitric oxide synthase; GSH, glutathione; IGF-1, insulin-like growth factor 1; Keap 1, Kelchlike ECH-associated protein 1; MDA, malondialdehyde; mTOR, mammalian target of rapamycin; Nrf2, NF-E2-related factor 2; ROS, reactive oxygen species; SIRT1, Sirtuin 1; SOD, superoxide dismutase.

Introduction

Moderate caloric restriction has been shown to decrease incidence of age-related diseases, including cardiovascular diseases, diabetes mellitus type 2 and cancer, and to increase lifespan (Weindruch and Sohal 1997; Masoro 2005; Mesquita et al. 2010; Ristow and Schmeisser 2011). The precise mechanism of anti-aging effect of caloric restriction (CR) is still unknown. Possible explanations are alteration of the insulin-like growth factor 1 (IGF-1) signaling (Bluher et al. 2003; Ristow and Schmeisser 2011), sirtuin 1 (SIRT1) and mammalian target of rapamycin (mTOR) (Colman et al. 2009), activation of the NF-E2-related factor 2/Kelchlike ECH-associated protein 1 (Nrf2/Keap1) (Birringer 2011), or inhibition of stress-induced apoptotic cell death (Cohen et al. 2004). The effect of CR on oxidative stress (OS) parameters in organism and hormesis might have a role in anti-aging mechanism of CR (Hunt et al. 2011).

One of theories of aging is the free radical theory. The concept of this theory is that if metabolic rate is increased, more reactive oxygen species (ROS) will be produced and

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hence it will cause cumulative cellular oxidative damage (Ristow and Zarse 2010). However, there is also the opinion that moderate CR actually acts like a low-level stress that induces ROS defense mechanism and increases stress resistance (Ristow and Zarse 2010).

Phenomenon that low dose of stressor has beneficial effects on cell, leads to increased stress resistance and promotes longevity is hormesis (Masoro 1998a,b; Radak et al. 2008; Le Bourg 2009; Marques et al. 2009). So far, it has been shown that applying low doses of stressor that has toxic effect at high doses induce adaptive response in different organisms, such as prokaryotes, yeasts, plants, intervertebrates and mammals. These effects were observed in humans as well (Calabrese et al. 2007; Gems and Partridge 2008; Colman et al. 2009; Marques et al. 2009). CR, irradiation, cold, heat, hyperbaric oxygen and chemicals such as paraquat or juglone have been proved as hormetic stressors *(*Marques et al. 2009; Hunt et al. 2011).

A lot of researches about hormesis were done, but the precise mechanism of hormesis is still unknown (Marques et al. 2009). Considering CR as a hormetic stressor, it is supposed that CR increases mitochondrial activity and ROS formation, and subsequent induction of ROS defense mechanisms (Birringer 2011; Ristow and Schmeisser 2011).

Although some experiments about aging and hormesis were carried out on rodents, most of them were done on invertebrate species such as *Drosophila melanogaster* or the nematode *Caenorhabditis elegans* (Le Bourg 2009). Therefore we have performed experiment on rats and examined the effects of different levels of chronic CR and acute fasting on OS parameters in rat liver and plasma, in order to better understand mechanism of hormetic effect of CR. Additionally, plasma corticosterone concentration was measured as an indicator of stress and activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma were measured as indicators of hepatocytes damage (Waite et al. 2002).

Also, to our knowledge, the effects of all different levels of CR which we had examined in this experiment, on oxidative stress parameters have not been analyzed in one study yet.

Materials and Methods

Ten weeks old male Wistar rats (200–220 g) used in this study were bred at Military Medical Academy in Belgrade. The rats were maintained under standard conditions, temperature 22 ± 2°C, relative humidity 50%, in 12 h light/dark cycle with free access to tap water. This study was performed respecting The European Council Directive (86/609/EC) and was approved by The Ethic Committee of Faculty of Medicine, University of Belgrade.

Forty-two animals were divided into following groups: control group ($n = 7$), four calorie-restricted groups receiving 80–90% (CR80-90, $n = 7$), 60–70% (CR60-70, $n = 7$), 40–50% (CR40-50, $n = 7$) and 20–30% (CR20-30, $n = 7$), of daily caloric needs during 5 weeks and acute fasting group (($n = 7$), one week without food intake).

Food restriction was calculated according to recommended food intake for ten weeks old rats which is 17 g of pelleted food. The diet consisted of maximum of 7% cellulose and minimum of 19% of proteins. Control group had recommended food intake.

After finishing the treatment, mild ether anesthesia was used and animals were sacrificed by decapitation. Liver samples and blood from the right side of the heart were collected for analyzing OS parameters, plasma corticosterone concentration, plasma AST and ALT activity.

Liver samples for biochemical analysis were homogenized on ice, in cold buffered 0.25 M sucrose medium (Serva Feinbiochemica, Heidelberg, NY, USA) with 10 mM phosphate buffer (pH 7.0) and 1 mM EDTA (Sigma-Aldrich Chemical Co., St. Louis, USA). The homogenates were centrifuged at 2000 × g for 15 min at 4°C. After centrifugation, sediments were dissolved in the sucrose medium and re-centrifuged as previously indicated. The supernatant was transferred into the tubes and centrifuged at 3200 × g for 30 min at 4°C. Obtained sediments were dissolved in deionized water. After 1 hour of incubation at 4°C, the samples were centrifuged again at 3000 × g for 15 min at 4°C, and supernatants were stored at –70°C. To determine proteins, the Lowry method with bovine serum albumin as the standard was used (Lowry et al. 1951).

Blood samples were collected from the right side of the heart into heparinized test tubes.

Activities of AST and ALT in plasma, as indicators of hepatocytes damage (Waite et al. 2002), were measured by spectrophotometer (Biosystems, BTS-330) and commercial kits (Sigma Chem. Co. St. Louis, USA). Plasma corticosterone concentration, as an indicator of stress (Waite et al. 2002), was measured by radioimmunoassay using tubes labeled with rabbit anti-corticosterone-antibodies.

Concentration of nitrites and nitrates (NO_x) in liver, indicators of NO oxide production (Cuzzocrea et al. 2003) was measured with Griess reagent. Reacting with NO_x, Griess reagent forms a purple colored compound and color intensity was measured spectrophotometrically (Biosystems, BTS-330) at 492 nm (Hibbs et al. 1989).

Total superoxide dismutase (SOD) activity in liver was measured spectrophotometrically (Biosystems, BTS-330) at 480 nm, as the percentage of inhibition of adrenaline autooxidation. After adding 10 mM of adrenaline (Sigma, St. Luis, USA), analysis was performed in the sodium carbonate buffer (50 mM, pH 10.2; Serva, Feinbiochemica, Heidelberg, New York) containing 0.1 mM ethylenediaminetetraacetic acid (Sigma, St. Luis, USA) (Sun and Zigman 1978). To

determine manganese superoxide dismutase (Mn-SOD) activity, samples were first treated with 8 mM potassium cyanide (Sigma, St. Luis, USA) and then analyzed as Sun and Zigman had described (Sun and Zigman 1978).

Lipid peroxidation was determined by measuring concentration of malondialdehyde (MDA), as Girroti and co-workers had described. Thiobarbituric acid reacting with MDA formed yellow colored compound and its absorbance was measured spectrophotometrically (Biosystems, BTS-330) at 533 nm (Girroti et al. 1991).

Glutathione (GSH) concentration was determined by using Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) in Tris-HCl buffer. DTNB in Tris-HCl buffer reacted with thiols groups in GSH, formed yellow colored nitro-thio-benzoate ion and color intensity was measured by spectrophotometer (Biosystems, BTS-330) at 412 nm (Anderson, 1986).

Statistical analysis

Data were presented as mean ± standard deviation. Differences between groups were tested by the one-way analysis of variance followed by the Fisher's post hoc test. $p < 0.05$ was considered statistically significant. SPSS 15.0 program was used for the statistical analysis.

Results

There was no significant difference in AST and ALT activity in plasma between control group, CR60-70 group and CR80-90 group ($p > 0.05$). On the other hand, AST and ALT activity in plasma was significantly higher in CR40-50, CR20-30 and acute fasting group in comparison to control group (Table 1).

Table 1. Effects of different levels of caloric restriction and fasting on ALT and AST activity in rat plasma

Group	ALT (U/l)	AST (U/l)
Control	36.4 ± 3.3	50.6 ± 4.1
CR80-90	34.7 ± 3.2	47.2 ± 3.9
CR60-70	38.1 ± 2.9	48.8 ± 2.9
CR40-50	62.7 ± 4.8*	62.8 ± 5[†]
CR20-30	67.9 ± 5.4*	68.3 ± 4.8*
Acute fasting	66.4 ± 5.7*	69.5 ± 4.3*

Value is mean ± standard deviation. AST, aspartate aminotransferase; ALT, alanine aminotransferase; CR20-30, calorie-restricted group receiving 20–30% of daily caloric needs; CR40-50, calorie-restricted group receiving 40–50% of daily caloric needs; CR60-70, calorie-restricted group receiving 60–70% of daily caloric needs; CR80-90, calorie-restricted group receiving 80–90% of daily caloric needs. * $p < 0.01$ compared to control group; † $p < 0.05$ compared to control group.

Corticosterone concentration in plasma was significantly higher in CR60-70, CR40-50, CR20-30 and acute fasting group than in control group (391.21 ± 16.77 ng/ml, 519.00 ± 11.83 ng/ml; 527.01 ± 26.69 ng/ml; 603.13 ± 17.09 ng/ml; respectively) ($p \leq 0.01$), while there was no significant difference between control group and CR80-90 group (282.07 ± 13.48 ng/ml; 289.02 ± 12.96 ng/ml; respectively). Corticosterone concentration was higher in CR40-50 and CR20-30 in comparison to CR60-70 ($p \leq 0.01$). Additionally, the highest level of plasma corticosterone was detected in acute fasting group (Figure 1).

There was no significant difference in MDA concentration between control group (2.86 ± 0.80 μmol/mg prot.), CR60-70 group (4.17 ± 0.69 μmol/mg prot.) and CR80-90 group (3.39 ± 0.86 μmol/mg prot.) ($p > 0.05$). In CR40-50, CR20-30 and acute fasting groups significantly higher MDA concentrations in liver (16.15 ± 0.93 μmol/mg prot.; 30.08 ± 4.76 μmol mg prot. and 27.00 ± 7.38 μmol/mg prot., respectively) were detected in comparison to MDA concentration in control group ($p \leq 0.01$). On the other hand, there is no significant difference in MDA liver concentration between CR20-30 group and acute fasting group ($p > 0.05$) (Figure 2).

In our study, NO_x concentration in liver was significantly higher in groups CR60-70 (7.45 ± 1.99 μmol/mg prot.; $p < 0.05$), CR40-50 (33.65 ± 11.05 μmol/mg prot.; $p < 0.01$), CR20-30 (35.79 ± 6.25 μmol/mg prot.; $p < 0.01$) and acute fasting group (35.05 ± 6.76 μmol/mg prot.; $p < 0.01$) than in control group (5.00 ± 1.79 μmol/mg prot.). In comparison to CR60-70 group, NOx concentration in liver in CR40-50, CR20-30 and acute fasting group was
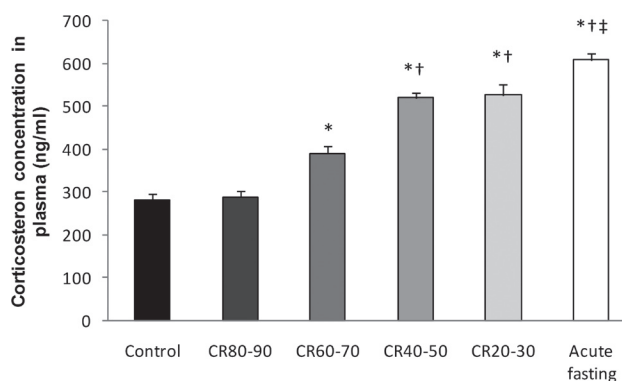
Figure 1. Effects of different levels of caloric restriction and acute fasting on corticosterone concentration in rat plasma. * $p < 0.01$ compared to control group; † $p < 0.01$ compared to CR60-70; ‡ $p < 0.01$ compared to CR20-30. CR20-30, calorie-restricted group receiving 20–30% of daily caloric needs; CR40-50, calorie-restricted group receiving 40–50% of daily caloric needs; CR60-70, calorie-restricted group receiving 60–70% of daily caloric needs; CR80-90, calorie-restricted group receiving 80–90% of daily caloric needs.
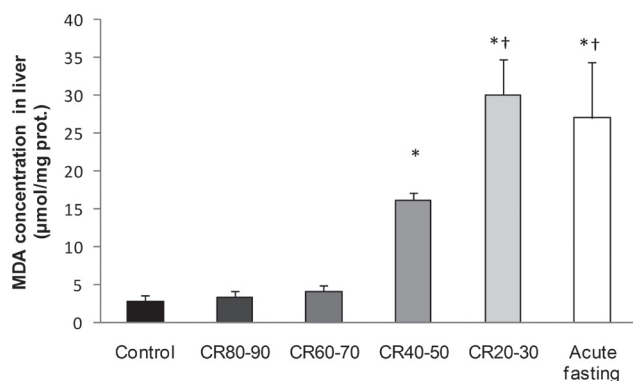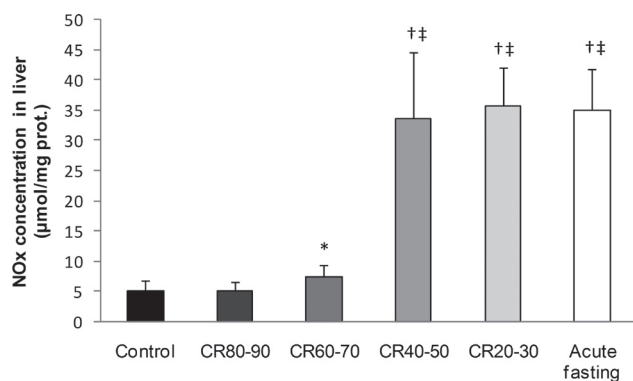
Figure 2. Effects of different levels of caloric restriction and acute fasting on malondialdehyde (MDA) concentration in rat liver. * $p < 0.01$ compared to control group; † $p < 0.01$ compared to CR40-50. (For abbreviations see Fig. 1).

significantly higher ($p < 0.01$). There was no significant difference, considering NOx level in liver, between CR80-90 group (5.19 ± 1.56 µmol/mg prot.) and control group ($p > 0.05$) (Figure 3).

SOD activity in liver was significantly lower in acute fasting group (1643.08 ± 80.69 U/mg prot.), CR20-30 (1487.98 ± 75.12 U/mg prot.) and CR40-50 group (1614.01 ± 99.46 U/mg prot.), comparing to control group (1892.34 ± 96.34 U/mg prot.; $p < 0.01$). Moreover, activity of the examined enzyme was the lowest in CR20-30 group. In contrast, when CR80-90 (1863.74 ± 82.08 U/mg prot.) and CR60-70 (1924.87 ± 91.32 U/mg prot.) groups were compared to control group no significant difference in SOD activity in liver was detected ($p > 0.05$) (Figure 4).

Activity of SOD isoform, Cu/Zn-SOD was changing in the same manner as total SOD activity (Figure 4).

Activity of mitochondrial SOD isoform, Mn-SOD in liver was significantly higher in CR60-70 group (193.45 ± 11.13 U/mg prot.) than in control group (124.08 ± 8.65 U/mg prot.; $p < 0.01$). Mn-SOD activity in liver was significantly lower in groups CR40-50 (98.02 ± 7.12 U/mg prot.), CR20-30 group (80.63 ± 8.05 U/mg prot.) and acute fasting group (92.74 ± 6.51 U/mg prot.), comparing to control group, but also comparing to CR60-70 group. There was no significant difference in Mn-SOD activity in liver between CR40-50 and acute fasting group ($p > 0.05$), as well as between CR40-50 and CR20-30 ($p > 0.05$) (Figure 5).

GSH concentration in liver was significantly higher in CR60-70 group (1.73 ± 0.10 nmol/mg prot.) than in control group (1.16 ± 0.09 nmol/mg prot.; $p < 0.01$). On the other hand, it was detected significant decrease in GSH concentration in CR20-30 group (0.54 ± 0.03 nmol/mg prot.) and acute fasting group (0.57 ± 0.04 nmol/mg prot.), in comparison to control group ($p < 0.01$). Comparing control group to CR80-90 group (1.08 ± 0.11 nmol/mg prot.) and to CR40-50 group (1.07 ± 0.09 nmol/mg prot.), no significant differences in GSH concentration in liver were found ($p > 0.05$) (Figure 6).

Discussion

In our study, ALT and AST activity in plasma (indicators of hepatocyte damage) was not increased in CR60-70 and CR80-90, which might indicate that moderete and mild caloric restriction had not caused hepatocytes damage. In contrast to, ALT and AST activity in plasma was increased in acute fasting group, CR20-30 and CR40-50 groups, which led us to conclusion that intensive CR and acute fasting had caused hepatic damage.
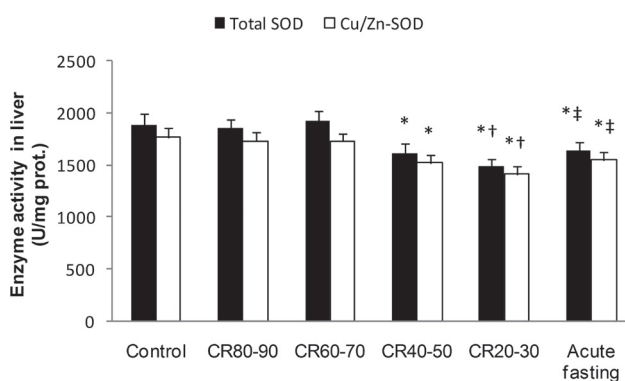

Figure 3. Effects of different levels of caloric restriction and acute fasting on concentration of nitrites and nitrates (NOx) concentration in rat liver. * $p < 0.05$ compared to control group; † $p < 0.01$ compared to control group; ‡ $p < 0.01$ compared to CR60-70. For abbreviations see Fig. 1).
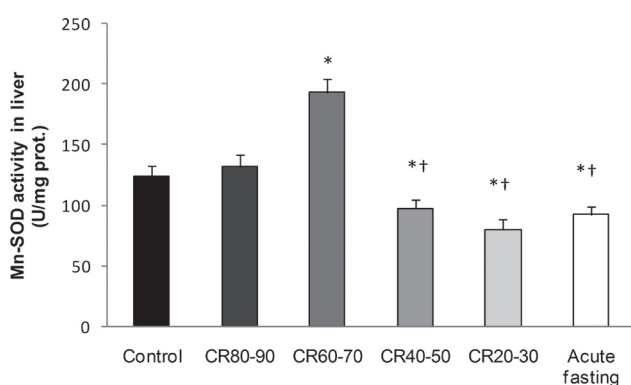

Figure 4. Effects of different levels of caloric restriction and acute fasting on total superoxide dismutase (SOD) and Cu/Zn-SOD activity in rat liver. * $p < 0.01$ compared to control group; † $p < 0.01$ compared to CR40-50; ‡ $p < 0.01$ compared to CR20-30. (For abbreviations see Fig. 1).

In present experiment, plasma corticosterone concentration increased when the calorie intake was restricted for 30% and more of recommended calorie intake, indicating that moderate and intensive chronic CR were stressful for organism. On the other hand, the highest concentration of corticosterone was in acute fasting group, meaning that acute fasting is a stronger stressor than chronic caloric restriction. Similar to our results, Chacón and co-workers reported increased plasma corticosterone level in rats after 4 weeks receiving 66% of recommended caloric needs (Chacón et al. 2005). Increased plasma corticosterone level was also reported in rats whose food intake during 14 days was reduced for 25% and 50%, comparing to control group (Ling and Bistrian 2009).

When animals in our study were fed with 50% and less of recommended daily caloric intake, MDA concentration in liver, as one of the measures of lipid peroxidation in liver, was progressively increasing. After one week of fasting, MDA liver level increased, while Abdelmegeed et al. reported that shorter period of fasting was not long enough to cause increase in lipid peroxidation, actually 36-h fasting did not significantly changed MDA concentration in mouse liver, comparing to control group (Abdelmegeed et al. 2009). After 12 months receiving 60% of the energy intake in control group, lipid peroxidation in rat muscle mitochondria even decreased for 46% in comparison to control group, suggesting protective role of this level of caloric restriction (Bevilacqua et al. 2005). On the other hand, after 18 months taking 40% less food than control group, lipid peroxidation was not changed in rat liver (Abdelmegeed et al. 2009). Similar to this result, in our study rats who were fed with 80–90% and 60–70% of daily recommended food intake did not have a significant changes in MDA liver concentration, comparing to control group.
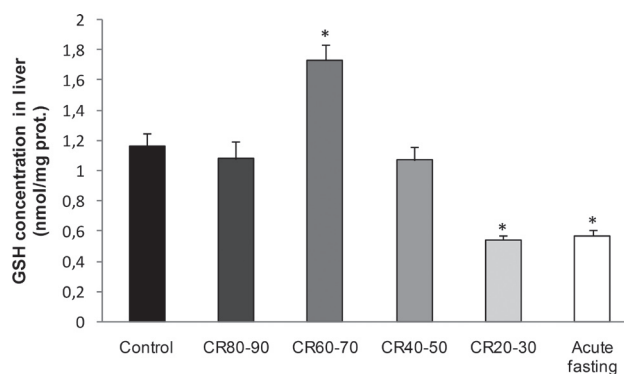
Figure 6. Effects of different levels of caloric restriction and acute fasting on glutathione (GSH) concentration in rat liver. * $p < 0.01$ compared to control group. (For abbreviations see Fig. 1).

NO_x concentration in liver significantly increased in CR60-70 group, when compared to control group. Since ALT and AST activity, as indicators of hepatocyte damage were not increased, explanation for NO_x increase may be that moderate CR induces endothelial nitric oxide synthase (eNOS) and consequently that low increase in NO level may have protective effect and may prevent age-related changes in blood vessels (Nisoli et al. 2005). Our results have shown that NO_x concentration in liver is significantly increased in rats with chronic CR for 50% and more, as well as in acute fasting group. These results may support suggestion that NO_x have important role in hepatocyte damage in intensive calorie restriction (Clemens 1999) since we have shown that AST and ALT activity are increased in groups CR40-50, CR20-30 and acute fasting group.

In our experiment taking 50% of recommended daily calorie intake or less significantly decreased SOD activity in liver. The explanation for this result might be that in contrast to mild and moderate chronic CR, intensive chronic CR as well as acute fasting, cause decrease in antioxidative liver capacity. SOD activity was lower in CR20-30 than in acute fasting group which means that chronic, intensive CR decreased antioxidative liver protection even more than acute fasting. Similar, SOD activity in liver was also decreased in mice, after 36-h fasting (Abdelmegeed et al. 2009). Although we did not reported any significant differences comparing SOD activity in group CR60-70 to SOD activity in control group, 18-month-old rats received 60% of the food given to control group had significantly increased hepatic SOD activity comparing to control group (Hamden et al. 2009). It was also reported that liver tissue of the rats fed with 60% of the diet of the rats fed ad libitum, at 21 and 28 months of age had significantly higher SOD activity (24% and 38%, respectively) comparing to rats fed ad libitum (Rao et al. 1990) which may also be explanation for protective effect of moderate CR.

Figure 5. Effects of different levels of caloric restriction and acute fasting on manganese superoxide dismutase (Mn-SOD) activity in rat liver. * $p < 0.01$ compared to control group; † $p < 0.01$ compared to CR60-70. (For abbreviations see Fig. 1).

In our study, the activity of SOD isoform, citosol enzyme Cu/Zn-SOD was changing in the same manner as activity of total SOD. On the other hand, the same changes were not reported considering activity of another SOD isoform, mitochondrial Mn-SOD. Our results showed that moderate CR (CR60-70) leads to increased Mn-SOD activity in liver. Since the most of ROS are produced in mitochondria, increased Mn-SOD activity may be one of hormetic mechanisms of moderate CR. On the other hand, intensive CR led to decreased Mn-SOD activity, which supports idea of CR as a hormetic factor. One of the explanations for this result might be that the enzyme was used for neutralizing ROS formed due to intensive CR. Another possible explanation for decreased Mn-SOD activity is nitrosilation of this enzyme and its inactivation by NO_x formed in intensive CR.

In our study, hepatic GSH concentration in CR60-70 was significantly higher than in control group. Similar to this result, Laganiere and Yu reported increase in hepatic GSH concentration in rats fed with 60% of the mean caloric intake of ad libitum fed group for 24 months (Laganiere and Yu 1989). Since GSH is antioxidant and important for neutralizing ROS (Pompella et al. 2003), increased GSH concentration may be also one of hormetic mechanisms of moderate CR. On the other hand, in CR20-30 and fasting group was detected significantly lower GSH concentration in comparison to control group. Abdelmegeed et al. (2009) also reported significant decrease in liver GSH level in mouse after 36-hour-fasting. These findings indicate that those levels of CR cause decrease in anti-oxidative liver capacity.

In conclusion, moderate CR triggers stress response, since it caused increase in plasma corticosterone concentration. This level of CR did not increase AST and ALT activities and MDA concentration in liver, which means that moderate CR does not cause hepatocyte damage and does not increase lipid peroxidation in liver. On the other hand, moderate CR increases hepatic antioxidant capacity, Mn-SOD activity and GSH concentration in liver, which, in our opinion, might have the role in anti-aging and hormetic effect of CR.

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