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Full Length Research Paper

Effect of different intensities of calorie restriction on liver and muscle mitochondrial oxidative stress

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The aim of the present study was to evaluate the effect of different levels of calorie restriction (CR) on liver and muscle mitochondrial oxidant production and antioxidant enzymes activity in adult rats. Male Sprague Dawley rats (n=28) were subjected to 25-75% CR for 15 days. H_2O_2 production was decreased in muscle mitochondria of 25-50% CR group. H_2O_2 production decreased in liver mitochondria of 25% CR group. The protein carbonyl content decreased in liver and muscle mitochondria of 25-50 % CR groups. TBARS levels were decreased in liver and muscle of (25-50%) CR groups. Superoxide dismutase and glutathione peroxidase activity was increased in liver mitochondria of 25% and 50% CR groups. Catalase activity was increased in liver and muscle of 25-50% CR groups. The 4-Hydroxy-2-neonal modified proteins increased in muscle of 75% CR rats. Results of the present study demonstrate that short term CR of 25-50% reduces the oxidative stress levels in muscle and liver mitochondria.

Key words: Calorie restriction, Mitochondria, Oxidative stress, Protein carbonyls and Antioxidant enzymes.

INTRODUCTION

Mitochondrial dysfunction is an important component of many of the pathologies associated with aging, such as type 2 diabetes mellitus, Alzheimer's disease, Parkinson's disease and some cancers (Weindruch and Walford 1988; Weindruch R and Sohal 1977). Indeed, mitochondria have been implicated overall in the aging process, although the mechanisms are not fully understood. One of the most widely accepted theories of aging, the oxidative stress theory, suggests that the aging process involves the accumulation of oxidative damage to mitochondria and other cellular components. Oxidative damage is induced by reactive oxygen species (ROS), produced primarily as a by-product of mitochondrial oxidative phosphorylation (Harman 1956; Sohal and Weindruch 1996), a process that is responsible for 85–90% of cellular oxygen consumption (Ames et al.,1995). As mitochondrial ROS can cause damage to mitochondrial DNA, proteins, and membrane lipids, a self-perpetuating and destructive cycle can ensue in which increased ROS production leads to incremental damage and further ROS production (Harman 1972).

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Calorie restriction (CR), without malnutrition, is a well known dietary intervention that consistently increases life span by delaying the aging process in a wide variety of animal species (Weindruch and Walford 1988; Weindruch R and Sohal 1977). The mechanisms underlying aging retardation by CR are poorly understood. However, it has been suggested that they may involve a decrease in cellular oxygen consumption and ROS production (Ramsey et al., 2000). The role that hypometabolism may play in the actions of CR is unclear, as several studies on whole body, organ-specific, and cellular oxygen consumption have yielded contradictory results (Ramsey et al., 2000). Other studies have demonstrated that CR mitigates age-associated increases in cellular ROS production and damage to cellular macromolecules in various tissues (Bevilacqua et al., 2004; Lopez-Torres et al., 2002).

The mechanisms that reduce ROS production during CR are still debated. Findings from studies have proposed that a decrease in mitochondrial proton leak or a lowered membrane potential that reduces resting oxygen consumption may be a possible explanation (Ramsey et al., 2000). However, in one of the reported study (Harper et al., 1998), proton leak is greater in hepatocytes of old (30 month) compared with young (3

month) mice. In skeletal muscle, a postmitotic tissue that is highly susceptible to oxidative damage, maximum leak-dependent respiration (state 4) is decreased by 40% CR in the short and medium term (2 wk, 2mo, and 6 mo) as well as in the long term (12 month) and by 33% CR at 23 month (Sohal et al.,1994). Most of these trials have been performed with 40% calorie restriction (CR) concerned with aged rats and it is not known whether similar conclusions can be made at restrictions that are higher or lower than this in adult rats. Therefore, the aim of the present study was to determine the effects of different intensities of calorie restriction (25-75%) on mitochondrial oxidative stress levels in adult rats.

MATERIALS AND METHODS

Animals and diet

Male albino Sprague-Dawly rats (n=28), weighing 198–205g were housed in cages with two animals per cage in a temperature (22°C), humidity and light control room. Animals were provided with standard rat chow diet and water *ad libitum*. All procedures and protocols used in the present study were approved by the Animal Care and Use Committee of the institute and followed the guidelines documented in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. Baseline food intake was measured over a period of 7 days to determine the amount of calorie required for restricted animals during the CR intervention phase. The rats were randomly divided into four groups [n=7] as control (without calorie restriction), 25% calorie restricted (25%CR), 50% calorie restricted (50% CR), and 75% calorie restricted (75% CR) groups. The experimental design was conducted for 15 days.

Collection of tissues and organs

On the 16th day, the animals were fasted overnight before they were killed by decapitation. Organs (liver, brain, heart, kidneys, spleen and muscles) were removed immediately cleaned of fat and weighed.

Isolation of mitochondria from liver and skeletal muscle

Mitochondria were isolated from liver and muscles by modified method of Bhattacharya et al., (1991). Briefly, the gastrocnemius muscles of each hind limb and liver were quickly removed and placed in ice-cold isolation medium (250 mM sucrose, 10 mM Tris HCl pH 7.4, 1 mM EGTA, 0.1% defatted BSA). The tissues were finely chopped and homogenates (10 % w/v) prepared in the isolation medium using glass-Teflon homogenizer at 4°C. The homogenate was centrifuged at 4000 × *g* for 15 min at 4°C, and the resulting supernatant was decanted into clean centrifuge tubes and centrifuged at 12000 × *g* for 30 min at 4°C. The supernatant was discarded, and the resultant pellet was resuspended in ice-cold isolation buffer and again centrifuged at 12000 ×*g*. The final pellet was resuspended in ice-cold suspension buffer (120 mM KCl, 20 mM Sucrose, 20 mM glucose, 10 mM KH_2P0_4, 5.0 mM HEPES, 2.0 mM $MgCl_2$, 1.0 mM EDTA, pH 7.2). Protein concentrations were determined using Folin's method (Lowry et al., 1951) with BSA as the standard.

Measurement of H_2O_2 production

Mitochondrial H_2O_2 production was determined using the p-hydroxyphenylacetate (PHPA) fluorometric assay (Hyslop and Sklar, 1984). Freshly isolated mitochondria (0.25 mg/ml) were added to 3 ml of 10 mM potassium phosphate buffer, pH 7.4, containing 154 mM KCl, 0.1 mM EGTA, 3mM $MgCl_2$, 500µg/assay *p*-hydroxyphenylacetate, and 4 units of horseradish peroxidase using 10 mM succinate or 10 mM pyruvate or 5 mM malate as substrates. Superoxide dismutase was not included in the reaction mixture. The increase in fluorescence at 37°C was monitored by a Perkin-Elmer LS 55 luminescence spectrometer. The excitation and emission wavelengths were 320 and 400 nm, respectively, and the final assay volume was 3 ml. Levels of H_2O_2 were expressed as nano mole of H_2O_2 per minute per milligram protein. Rates were determined by converting fluorescence readings using a standard curve generated over a range of H_2O_2 concentrations.

Determination of protein Carbonyls

Mitochondrial reactive carbonyl derivative was determined by the 2, 4-dinitrophenylhydrazine (DNPH) method (Reznick and Packer, 1994). Briefly, 1ml of mitochondrial suspension in two separate tubes, one treated with either 2.5 M HCl alone (control) and other with 2.5 M HCl containing 10 mM DNPH incubated at 27°C for 1 h in dark. After the incubation the mixture was treated with 20% TCA and centrifuged to discard the supernatant. Finally the precipitates were washed with an ethanol-ethyl acetate (1:1) mixture three times to remove free DNPH. The final precipitate was dissolved in 2 ml of 6 M guanidine hydrochloride solution and was incubated for 10 min at 37°C. The absorbance was measured at 355 nm, and the carbonyl content was obtained as µ mole carbonyl per milligram protein using a molar extinction coefficient of 22,000 $M^{-1}cm^{-1}$.

Determination of lipid peroxidation

Lipid peroxidation levels or thiobarbituric acid reactive substances (TBARS) were determined as malondialdehyde-thiobarbituric acid adducts according to the modified method (Ohkawa et al., 1979). Using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ and expressed as µmole TBARS per min per milligram protein.

Determination of lipid hydro peroxides by ferrous oxidation in xylenol orange reagent assay [FOX-1]

Muscle and liver hydroperoxides were estimated according to the reported method of Wolff (2004). This method is extremely sensitive [ε560 (H_2O_2) = 2.2 x 10^5 M^{-1} cm $^{-1}$] to measure low levels of hydroperoxide. Briefly, 50µl of sample was added to 950µl of ferrous oxidation in xylenol orange reagent (FOX) containing 100 µM xylenol orange, 250µM ammonium ferrous sulphate, 100 mM sorbitol, and 25mM H_2SO_4. The reaction mixture was vortexed and incubated for 30 min at 27°C. The colour developed was read at 560 nm.

Nitric Oxide [NO] production assay

NO production was measured by the accumulation of nitrites present in mitochondrial suspension, by the reported method (Green et al., 1982). Absorbance was measured using an ELISA reader (Molecular Devices, versa Max) using $NaNO_2$ (1-20) µm as the standard. The results are expressed as µmol NO per mg protein.

Measurement of antioxidant enzyme activities

The Super Oxide Dismutase activity (SOD; EC1.15.1.1) was measured spectrophotometrically using commercially available kit [RANDOX Laboratories, Crumlin, UK]. One unit activity of SOD

Table1. Effect of 15 days of gradual calorie restriction on total body, muscle and organ (heart, kidney, liver, spleen) weight

Variable	Control Rats	Calorie restricted rats		
		25%	50%	75%
Initial body weight(g)	200 ±18	198±12	202±18	200±20 $_{NS}$
Final body weight(g)	250 ±10	175±14**	172±11.6**	152±18***
Gastrocnemius (g)	2.6±0.3	2.7±0.4	2.6±0.4	2.6± 0.3 $_{NS}$
Heart (g)	0.95±0.02	0.93±0.02	0.93±0.1	0.92±0.1$_{NS}$
Kidney(g)	1.66±0.02	1.66±0.04	1.59±0.09	1.55±0.03$_{NS}$
Liver(g)	10.9±0.4	8.83±0.1**	8.11±0.3**	6.07±0.1***
Spleen(g)	0.55±0.03	0.52±0.01	0.51±0.01	0.48±0.1
Brain(g)	1.7±0.1	1.6±0.02	1.7±0.1	1.6±0.3 ^{NS}

Data are reported as mean ± SEM (n=7). * P<0.05, ** P<0.01, ***P<0.001, NS, Not significant difference from control group.

was determined by monitoring the 50% inhibition of the rate of reduction of 2-(4-Iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium under the conditions of the assay as mentioned in kits protocol. Catalase activity **(**CAT; EC 1.11.1.6) was assayed according to the reported method (Aebi, 1984). One unit of catalase activity was defined as the amount of enzyme required to decompose 1 µ mol of hydrogen peroxide (H_2O_2) per minute. Catalase activity was expressed as µmole of hydrogen peroxide reduced/ min/mg protein.

Glutathione Peroxidase Activity **(**GPX; EC 1.11.1.9) was measured according to the reported method (Rotruck et al., 1973) using H_2O_2 as the substrate and decrease in absorbance was measured at 340 nm. Enzyme activity was expressed as µmol NADPH oxidized/min/mg protein.

Detection of 4-hydroxy-2-nonenal-modified proteins

The detection of 4-hydroxy-2-nonenal (HNE) modified proteins according to the method of Judge et al. (2005). Mitochondrial proteins (30 µg) were separated using 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels under denaturing conditions and then transferred to nitrocellulose. The membranes were blocked in PBS/Tween-20 and incubated with rabbit anti-HNE antiserum (Sigma aldrich, TX) at a 1:250 dilution overnight. The next morning, the membranes were washed in PBS/0.05% Tween-20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:1000 dilution for 1 h. ECL detection reagents from Amersham (Amersham Biosciences, NEA) were used to generate a chemiluminescent signal and bands were visualized by exposing the membranes to Hyperfilm-ECL (Amersham Biosciences, NEA). Blots were analyzed using Kodak 1D Image Analysis software (Amersham Biosciences, NEA).

Statistical analysis

Values are expressed as means ± SEM, n=7. Statistical analysis was performed with commercially available software (SPSS version 15.0). Data were evaluated for significance by one - way ANOVA followed by student Newman-keuls test for multiple group comparison. Differences of P<0.05 were considered significant.

RESULTS

Body weight & organ weight

Initial body weight of the rats did not differ between experimental groups at the beginning of treatment (Table 1). Whereas control rats gained weight (+25%), all calorie restricted rats progressively lost body weight (12-24%), as the intensity of CR increased. At any given percentage of CR, the gastrocnemius muscle weight, heart, and brain masses remained unchanged whereas, maximum change in weight was observed in case of liver (Table 1). Liver mass and the liver-to-body weight ratio decreased linearly with the intensity of CR with a final loss of 56%. The kidney weight was slightly decreased in respect to control. The wet weight of spleen also decreased in line with intensity of CR but remaining unaffected relative to body weight.

H_2O_2 production

H_2O_2 production was decreased significantly (P<0.01) in liver and muscle mitochondria of 25% CR group compared with control, whereas H_2O_2 production was significantly (P<0.05) decreased in liver mitochondria of 50% CR group compared with controls. The H_2O_2 production was significantly (P<0.05) increased in muscle mitochondria of 75% CR group compared with control (Figure.1).

TBARS content and Protein carbonyls

Protein carbonyls have been used as a marker of protein oxidation. Protein carbonyls significantly decreased in both muscle (P<0.05) and liver (P<0.01) of 25%, 50% CR

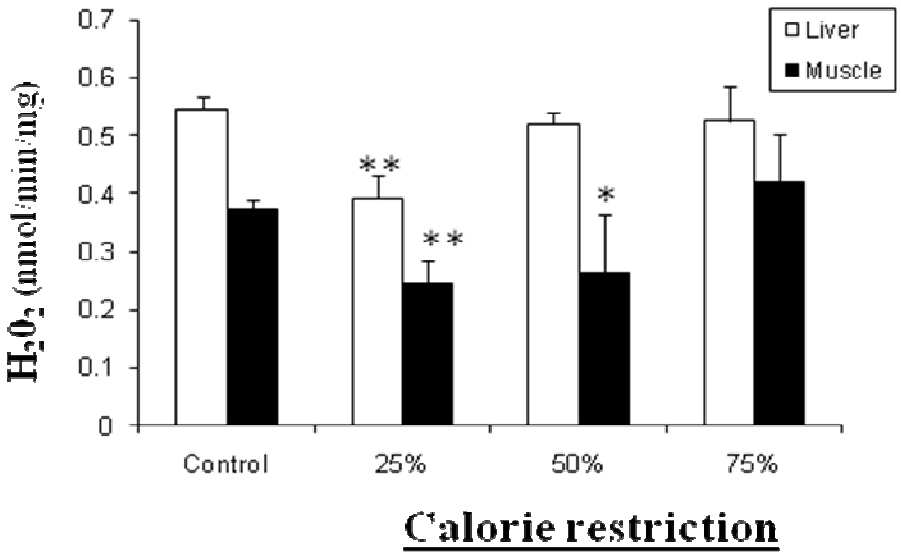

Figure.1. Effect of CR on H_2O2 production in liver and muscle mitochondria. Oxidant production was measured using a fluorometric p-hydroxy phenylacetate assay. Data are reported as mean ± SEM (n=7), *p<0.05, *p <0.01 difference from control group.

Table 2. Effect of calorie restriction on oxidative stress markers

Organs		Control	Calorie Restricted Rats		
			25%	50%	75%
Liver	TBARS	2 ±0.2	1.1 ±0.4*	1.2±0.2*	2.1±0.2
	Hydroperoxides	0.32±0.3	0.24±0.2	0.29±0.1	0.41±0.5
	NO	85±2.4	77±3.6	82±1.2	89±1.0
Muscle					
	TBARS	1.4 ±0.2	1.9 ± 0.2*	0.92±0.4*	1.68 ± 0.2
	Hydroperoxides	1.5±0.01	1.7±0.02	1.7±0.02	1.9±0.02
	NO	54±1.2	48±2.1	56±1.6	60±2.8

Oxidative stress parameters are expressed as µmol/min/mg protein. Data are reported as mean ± SEM (n =7) * P<0.05 difference from control group.

groups, when compared with control and there was no significant difference was observed in 75% CR group (Figure.2). TBARS content significantly decreased in both muscle (P<0.05) and liver (P<0.05) mitochondria of 25%, 50% CR groups. Whereas, slight increase was observed in muscle TBARS content of 75% CR group in comparison to control (Table 2).

Nitric oxide production

No significant difference was observed in both liver and muscle mitochondrial NO production of CR groups when compared with control (Table 2).

Hydroperoxides [LHP]

There was no difference in lipid hydroperoxides content in both muscle and liver mitochondrial of 25-50% CR rats compared with control rats and there was no significant difference observed in case of 75% CR rats. Whereas, significant (P<0.05) increase was observed in muscle mitochondria of 75% CR group in comparison with control (Table 2).

Antioxidant enzyme activities

The effect of CR on the activities of liver and skeletal muscle mitochondria SOD, CAT and GPX is summarized

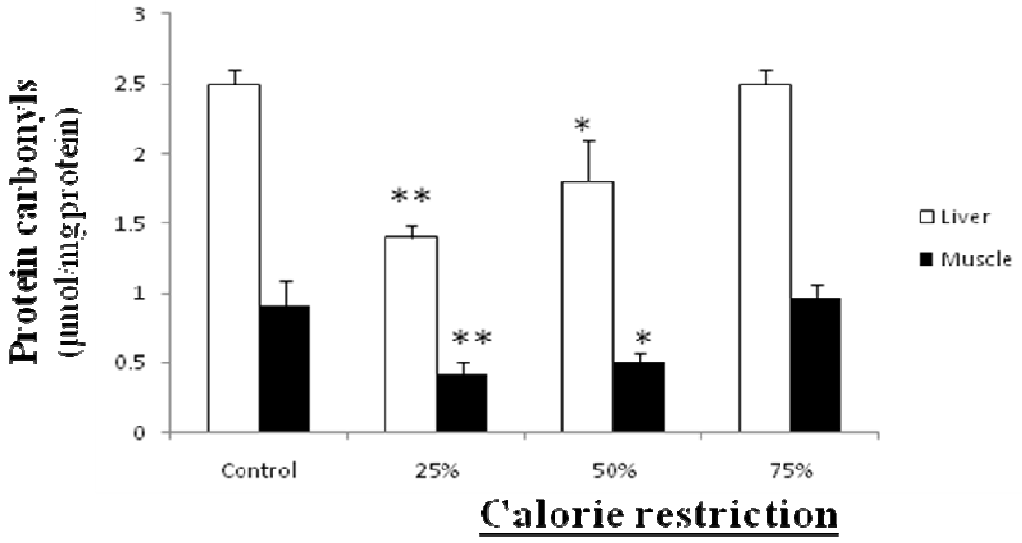

Figure. 2 Effect of CR on protein carbonyl content in liver and muscle mitochondria. Data are reported as mean ± SEM (n=7), *p<0.05, **p <0.01 difference from control group.

in Table 3. SOD activity was increased significantly in liver mitochondria of 25% (P<0.01) and 50% (P<0.05) CR rats in comparison with control whereas, in 75% CR rats the activity was decreased significantly when compared with control. No significant difference was observed in muscle mitochondrial SOD activity of CR rats compared to control. The catalase activity was increased significantly in muscle (P<0.01) mitochondria of 25-50 % CR group compared with control. However, there was no significant change was observed in liver mitochondrial catalase activity of 25-50% CR group in comparison with control. The GPX activity was increased significantly (P<0.01) in liver of 25-50% CR group compared to control group. GPX activity was increased (P<0.05) significantly in muscle mitochondria of 25 % CR group in comparison to control. No change was observed in both liver and muscle of 75% CR group in comparison to control.

4-hydroxy-2-nonenal content

No differences in the amount of HNE modified proteins in the liver and muscle of 25-50% CR rats in comparison to control. The amount of HNE modified proteins increased (P<0.05) in muscle mitochondria of 75% CR rats. No change was observed in the amount of liver mitochondrial HNE modified proteins of 75% CR group (Figure.3).

DISCUSSION

The objective of the present study was to investigate the effect of short-term different levels of calorie restriction on skeletal muscle and liver mitochondrial oxidant production and antioxidant enzymes. The results demonstrated that 15 days of CR has differential effect on liver and muscle mitochondrial SOD, CAT and GPX activities and TBARS content, protein carbonyl content, and H_2O_2 production.

There was 25% weight gain in control animals over a period of 15 days in comparison to their initial weight, whereas proportional weight loss was observed in 25-75% CR rats. The effect of CR in reduction of weight of different organs is highly variable and is not proportional to reductions in body weight or energy intake. The maximum change in weight was observed in case of liver whereas are brain, heart and kidney were unaffected and similar results are reported by different investigators Similar result has been reported by earlier studies (Ramsey et al.2004; Gomi and Matsuo 1998; Dumas et al. 2004). Initial decrease in liver weight may be attributable to reductions in liver glycogen stores.

Our findings indicate that H_2O_2 production decreased in 25-50% CR in both muscle and liver mitochondria. Bevilacqua et al. (2004) reported that H_2O_2 production was decreased by 53% and 57% in muscle mitocondria after 2 week and 2 month of CR. Hagopian et al. (2005) reported that the H_2O_2 production was decreased significantly in liver mitochondria after 18 month of CR and no difference was observed at 12 mont h of CR. The differences between liver and skeletal muscle H_2O_2 production may reflect differences in duration and degree of CR and differences between mitotic (liver) and postmitotic (skeletal muscle) tissues.

Several other studies (Zainal et al. 2000; Matthias et al.2004; Burrin et al. 1989) have documented CR-

Table 3. Effect of calorie restriction on antioxidant enzyme activities in liver and muscle mitochondria

Organ	Enzyme activities	Control	Calorie Restricted Rats		
			25%	50%	75%
Liver	SOD	0.59 ±0.07	0.86±0.06**	0.71±0.04*	0.46±0.04
	Catalase	75.52 ±0.3	81.27±0.2	75.33±0.07	63.84±0.5
	GPx	119.46±0.4	142.85±0.9**	141.65±0.6**	116.74±0.9
Muscle	SOD	2.36 ±0.05	2.07 ± 0.02	2.72±0.08	1.28 ± 0.08
	Catalase	43.51±1.3	73.73± 0.6**	81.49± 0.4**	41.57±0.9
	GPx	159.60±0.24	183.25±0.4*	132.58±0.2	125.72±0.9

Enzyme activities are expressed as μmol/min/mg protein. Data are reported as mean ± SEM. (n =7) * P<0.05, ** P<0.01 difference from control group.

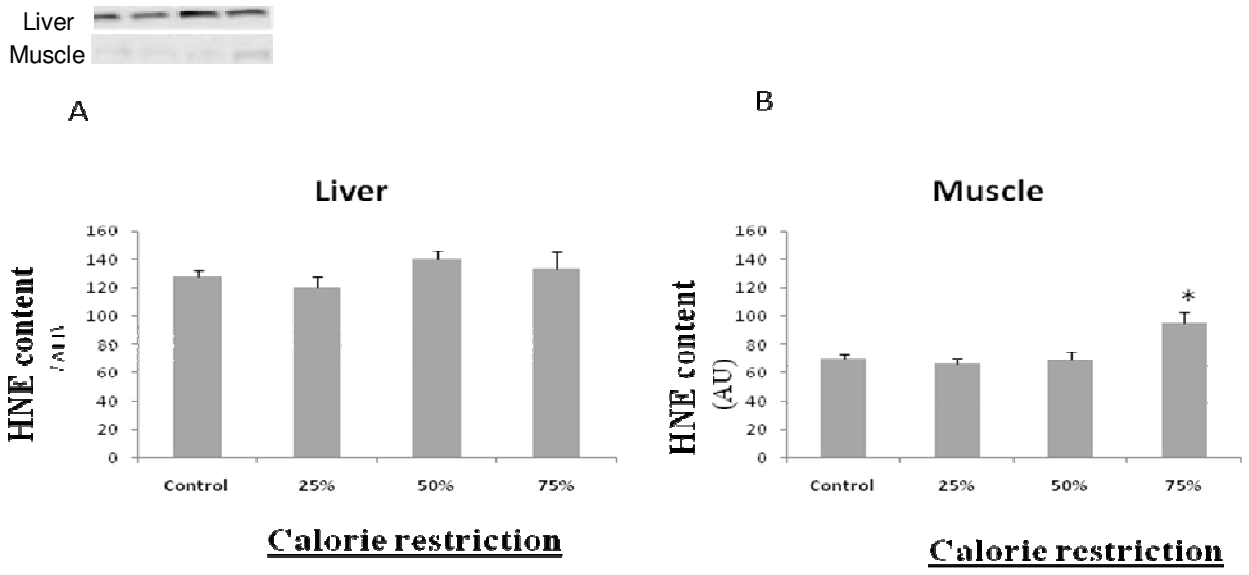

Figure 3. . Western blot analysis of 4-hydroxy-2-nonenal-modified proteins (HNE) in liver (A) and muscle mitochondria (B) of CR and control rats. Data are reported as means ± SEM (n=7), *p <0.05 difference from control group.

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induced decreases in mitochondrial protein oxidation in a variety of tissues. Our hypothesis is that reduction in H_2O_2 production could contribute significantly to low oxidative damage to mitochondrial proteins. Mitochondria are the primary cellular source of endogenous ROS. Therefore, due to their location, mitochondrial proteins may be the primary target of the protection conferred by the CR. TBARS have been used as a marker for lipid peroxidation. In an attempt to explain the reduction in lipid peroxidation in both muscle and liver mitochondria after CR, we evaluated antioxidant enzyme activity. The elevation of SOD, CAT, and GPX activities may contribute to the reduction in lipid peroxidation.

SOD converts superoxide anion into H_20_2 and O_2, whereas CAT and GPX reduce H_2O_2 to H_20, resulting in the detoxification of free radicals (Paoletti and Mocali, 1990). Thus elevation of SOD, CAT, and GPX activities may contribute to the decrease of protein carbonyl content. Another mechanism may be CR-induced reduction of energy expenditure, consequently leading to lowered ROS (Ramsey et al. 2000). In addition, CR may lead to reduced mitochondrial oxy radical production and increased expression of cytoprotective stress proteins,

which may suppress oxyradical production and stabilize cellular homeostasis (Mattson 2000). Several other studies (Pamplona et al. 2002, Radak et al. 2002) have documented CR-induced decreases in mitochondrial protein oxidation in a variety of tissues.

The 4-hydroxy-2-nonenal (HNE) is an end product of lipid peroxidation that is highly reactive with other biological molecules, including proteins. HNE exerts numerous effects, including inhibition of protein and DNA synthesis and enzyme inactivation, and is believed to play a major role in oxidative stress-induced cellular dysfunction (Judge et al. 2005). Here we also observed the differential response of calorie restriction and starvation. The 75% CR is considered as starvation response, it showed negative response with respect to some markers of oxidative stress. Starvation can be viewed as a most extreme form of caloric restriction and it is not linked with life span extension (Matthias et al. 2004).

CONCLUSION

Result of the present study suggests that short term with different intensities (25-75%) of CR has shown to differentially affect the skeletal muscle and liver mitochondrial oxidant production and the accumulation of mitochondrial damage. Short term CR of 25-50% reduces the oxidative stress levels in both muscle and liver mitochondria. Further studies are required to fully characterize the response of different tissues with short term CR on mitochondrial oxidant production.

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