Attenuation of Tissue Oxidative Stress by Dietary Restriction in Rats on Simulated Microgravity

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Abstract. Introduction: Physiologic alterations caused by oxidative stress can be assessed by measuring tissue malondialdehyde (MDA) levels, a biomarker for oxidative stress. The goal of this study is to determine the consequences of a twenty percent caloric restriction on the increased oxidative stress documented in tissues from rats exposed to simulated microgravity. Materials/Methods: Three groups of male SD rats (N=6 in each group) were used: Group 1, control; Group 2, food restricted (20% less food than control); and Group 3, food restricted with HLS. Group 3 was suspended after one week on the HLS-restricted diet and maintained for 14 days. Tissues harvested on day 14 were measured for MDA levels. Results: The body weight gain of Group 2 and Group 3 was reduced as compared to that of Group 1 (p<0.05) with no significant changes in water intakes. MDA levels in Group 2 were not different from those of the control group and were elevated only in liver tissues (p<0.05). In Group 3, MDA levels in the heart, liver, brain, and testes were significantly elevated (p<0.05) compared to the levels of Groups 1 and 2. Conclusions: Food restriction alleviated tissue oxidative response in all tissues except for the liver. Excessive stress resulting from HLS appeared to have been minimized by dietary restriction in all tissues except for the heart, liver, brain, and testes.

Key words: hindlimb suspension, caloric restriction, simulated microgravity, tissue oxidative stress

Introduction

Caloric restriction prolongs lifespan through a reduction in oxidative stress [1-5]. The mechanism by which this occurs in both humans and animals is attributed to a reduction in mitochondrial oxidant production and an increase in mitochondrial bioefficiency [6-8]. Physiologic alterations caused by oxidative stress can be assessed by measuring tissue malondialdehyde (MDA) levels, a biomarker for oxidative stress. Recently, Barkha et al. [9] reported that caloric restriction increases lipid peroxidation while decreasing mitochondrial bioenergetic efficiency in a mouse model of amyotrophic lateral sclerosis (ALS). Extrapolation of these findings in ALS patients would contraindicate the benefits of caloric restriction. This effect appears to be due to an increase in basal oxidative stress resulting from the over-expression of the mSOD1 protein [10, 11]. A subsequent analysis of the reports of Barkha et al. [9] indicates that the effects of short-term caloric restriction depend on the tissues examined, the age of the animals, the duration of restriction, the magnitude of restriction and the type of rodent species being analyzed. Previous studies from our laboratory used the NASA-validated rat model of simulated microgravity and repeatedly confirmed the increase of malondialdehyde, a biomarker of oxidative stress and an end-product of lipid peroxidation in various tissues [12-18]. Whether introduction of calorie restriction will alleviate this induced stress in the tissues from animals that are exposed to simulated microgravity has not previously been studied. In this study, we have determined the specific consequences of a twenty percent restriction of the ad libitum diet on the oxidative stress in Sprague-Dawley rats exposed to simulated microgravity.
Materials and Methods

All experiments were conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and protocols were approved by the UAMS Animal Care and Use Committee. Eighteen male 200 to 250 g Sprague-Dawley rats were divided into three Groups (n = 6). The initial week was a priming week, with six rats per cage and with unrestricted access to food and water. At the end of each day, the weight of each rat, food eaten, and water consumed were measured. At the beginning of the second week, Groups 2 and 3 (n = 6 each) were placed on 20% dietary reduction as measured with regard to the amount of food consumed by the control group. At the beginning of the third week, Group 3 (n = 6) was hindlimb-suspended by the tail to simulate exposure to micro-gravity. In Groups 2 and 3, food eaten and water consumed were replaced, and body weight was measured daily.

**Hindlimb Suspension (HLS).** HLS was achieved using a modification of the Morey-Holton tail-suspension model [19]. HLS was accomplished with a tail harness constructed by looping a 0.5 x 10-inch Skin-Trac (Zimmer, Inc., Charlotte, NC) orthopedic foam strip around a pulley that could travel along a bar that traverses the length of the cage. The adhesive surfaces along the remainder of the Skin-Trac strip were applied to the long axis on opposite sides of the tail, creating a *tail-sandwich*. This sandwich was encircled by a bias-cut orthopedic stockinette and secured with one-inch glass zip-reinforced strapping tape at the base and tip of the tail. This construction does not interfere with the animal’s ability to use its tail to maintain its core body temperature. Control values were recorded for seven days before suspending the animals at a 30° angle to the cage floor. Twenty-four hours before sacrifice, the animals were deprived of food but not water. At sacrifice, rats were anesthetized with ketamine hydrochloride and acyl promazine (0.1 ml/100 g body weight) and euthanized by decapitation and exsanguination. Brain, heart, lung, liver, kidney, pancreas, testes, skeletal muscle, and small intestine were harvested. Each organ was washed in ice-cold normal saline solution. After determining the weight of each organ, each sample was homogenized in 20 mM phosphate buffer (pH = 7.4; tissue/buffer ratio, 1/10 w/v), and 10 µL of 0.5 M butylated hydroxytoluene tolune (BHT) was added per 1.0 ml of homogenate to prevent sample oxidation.

**MDA Assay.** The MDA assay was conducted by the method reported by Esterbauer *et al* [20] using the LPO-586 method (Bioxytech LPO-586, R&D Systems, Minneapolis, MN 55413). Harvested tissues were washed in ice-cold NaCl solution (9 gm/L). After excising 0.4 to 0.5 grams from each tissue, each sample was homogenized in 20 mM phosphate buffer, pH = 7.4 (tissue to buffer ratio, 1:10 w/v). 10 µL of 0.5 M butylated hydroxytoluene (BHT) per ml of homogenate had been added to prevent further sample oxidation. The homogenate was centrifuged at 4,000 g at 4 °C for 10 minutes. 200 µL of supernatant from each homogenate was used to analyze the MDA levels.

Decomposition of polyunsaturated fatty acid peroxides produces MDA and 4-hydroxyalkenals (HAE). Measurement of MDA was based on the reaction of a chromogenic reagent, 

Table 1. Mean food intake per week ± SEM (grams). Mean food intake measured in grams/day/group ± standard error of mean. Food was weighed and measured at twenty-four hour intervals over the course of twenty-one days.

<table>
<thead>
<tr>
<th>Week</th>
<th>Control</th>
<th>Dietary Restricted</th>
<th>Dietary Restricted &amp; HLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>62.40 ± 2.64</td>
<td>60.00 ± 2.6</td>
<td>62.00 ± 3.17</td>
</tr>
<tr>
<td>2</td>
<td>66.75 ± 1.06</td>
<td>54.00 ± 0 *</td>
<td>54.00 ± 0 *</td>
</tr>
<tr>
<td>3</td>
<td>69.56 ± 2.30</td>
<td>54.00 ± 0 *</td>
<td>54.00 ± 0 *</td>
</tr>
</tbody>
</table>

* p-value < 0.05, significantly less than control
N-methyl-2-phenylindole [R1], with MDA at 24 °C. One molecule of MDA interacts with 2 molecules of R1 to yield a stable chromophore with maximal absorbance at 586 nm, which is stable for up to one hour at room temperature. The net absorbance at 586 nm yields a linear function of MDA ranging from 0 to 20 µM. The detection limit is 0.1 µM of MDA. The final MDA content is expressed as µM of MDA per mg. protein.

**Protein Assay.** Protein concentrations were measured using the Biuret Method (Bio-Rad Laboratories) as described by Bradford [21]. Known concentrations of bovine serum albumin were used for standards.

**Statistics.** Results were calculated as means ± standard error of the mean (SEM). Statistical significance was determined by One-Way ANOVA. A value of p < 0.05 was considered significant.

**Results.** Neither food intake nor body weight differed significantly after the first week (Table 1 and Table 2). Food intake was intentionally reduced by twenty percent for Groups 2 and 3 during the second and third weeks. At the end of three-week period, the body weight gain of Group 1 was 70 g as compared to 30 g for Group 2 and -2 g for Group 3, while during those periods the mean food intake was significantly lower (p < 0.05) in Groups 2 and 3 than in the control group. Water intake did not vary significantly among the Groups. As anticipated, Groups 2 and 3 lost body weight during the second and third weeks as compared to the first week. This was anticipated due to partial reduction of caloric restriction as well as the stress induction resulting from both calorie restriction as well as hindlimb suspension.

MDA levels were significantly higher in the livers of Group 2 (diet-restricted rats) than in the livers of Group 1 (control) or Group 3 (restricted and suspended rats) after the second week (Table 3). This was presumably the direct result of dietary restriction, since suspension did not cause the anticipated increase from calorie restriction. MDA levels in the hearts, brains, and testes were significantly higher in the restricted and suspended animals at the end of the experiment, presumably due to suspension, since there was no significant difference in MDA levels between the control and the restricted rats in these organs. There was no significant difference in MDA levels in the lungs, pancreas, kidneys, small intestines, or muscles at the end of the experiment as a result of suspension between the three Groups. This result was unanticipated based on our previously published results [12-18], suggesting that calorie restriction helped to reduce the elevation of oxidative stress biomarkers in the tissues of rats subjected to hindlimb suspension.

**Discussion**

The positive effects of caloric restriction presumably act through a reduction in tissue oxidative stress and are attributed to a reduction in mitochondrial oxidant production. This study investigated the effect of a twenty percent dietary restriction on the oxidative stress induced in tissues under simulated microgravity [22] using NASA’s hindlimb unloaded rat model [19]. This was done

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**Table 2. Mean weight at end of week ± SEM (grams).** Mean body weight measured in grams/rat/week ± standard error of mean. The weights of the rats were measured at regular twenty-four hour intervals. The weights per group were averaged at the end of each week and are represented in this table.

<table>
<thead>
<tr>
<th>Week</th>
<th>Control</th>
<th>Dietary Restricted</th>
<th>Dietary Restricted &amp; HLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>243.33 ± 11.11</td>
<td>230.67 ± 09.63</td>
<td>246.00 ± 07.03</td>
</tr>
<tr>
<td>2</td>
<td>282.00 ± 13.33</td>
<td>250.67 ± 14.45</td>
<td>259.33 ± 07.52</td>
</tr>
<tr>
<td>3</td>
<td>313.00 ± 13.22*</td>
<td>264.67 ± 17.51</td>
<td>244.67 ± 07.52</td>
</tr>
</tbody>
</table>

* p-value < 0.05, significantly greater from week 1
in an attempt to demonstrate a beneficial effect of calorie restriction or use of healthy diet for those anticipating travel in a weightless or microgravity environment. A dietary restriction was implemented in an attempt to counteract the increased oxidative stress associated with exposure to microgravity [22], as well as with the aging process and several human disease conditions [5, 9, 11, 23]. The twenty percent dietary restriction imposed on these animals significantly increased MDA levels in their livers, as well as in the hearts, testes, and brains of calorie-restricted and hindlimb-suspended animals at the end of the three-week experiment. Dietary restriction is known to activate fatty acid synthesis in the liver [20]. Liver MDA levels were not significantly altered in calorie-restricted animals following suspension. Hence, the increased MDA levels in the liver were probably due to the restriction alone. The twenty percent restriction had no significant influence on MDA levels in other tissues harvested (Table 3). However, HLS significantly increased MDA levels in the heart, brain, and testes (Table 3). This was an anticipated change from our previously published data [12-18], and calorie restriction did not minimize the oxidative effects in these tissues.

Oxidative stress is known to be a marker of neurodegenerative diseases such as Alzheimer’s and Parkinson’s [9, 11, 24]. The mechanism of this effect could result from an increase in membrane lipid peroxidation with associated increases in MDA levels. In the heart, microgravity (with the loss of forces that assist in the movement of blood) may influence oxidative stress, considered a marker of heart failure [23]. Microgravity in the testes results in induction of apoptosis as well as decreased sperm count and motility [25]. It is possible that this effect is related to an increase in MDA levels and oxidative stress. In addition, the testes in rats can reenter the abdominopelvic cavity in the suspended rat, and the excess body heat might contribute to the increased MDA (stress) levels noted in this study.

Thus, it appears that a twenty percent reduction in food intake for two weeks duration is sufficient to significantly alter cellular membrane oxidative stress in all tissues in food-restricted Groups except in the liver, where the reduction alone resulted in a significant increase according to hepatic malondialdehyde levels. The subsequent influence of simulated microgravity, which has been shown to increase oxidative stress above control levels in all tissues studied, did not further increase hepatic stress levels. This effect could be due to the caloric restriction exerting a protective effect during this time. The addition of simulated microgravity only

### Table 3. Effect of ad libitum diet, diet restriction, and diet restriction plus hind limb suspension on mean MDA levels ± SEM. MDA expressed in µm/mg protein.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>Dietary Restricted</th>
<th>DR &amp; HLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>.851 ± .039</td>
<td>0.876 ± .052</td>
<td>1.341 ± .200 **</td>
</tr>
<tr>
<td>Lung</td>
<td>1.12 ± .095</td>
<td>1.040 ± .097</td>
<td>0.980 ± .089</td>
</tr>
<tr>
<td>Pancreas</td>
<td>.547 ± .069</td>
<td>0.633 ± .074</td>
<td>0.602 ± .118</td>
</tr>
<tr>
<td>Liver</td>
<td>.623 ± .104</td>
<td>1.328 ± .243 *</td>
<td>1.196 ± .179 *</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.00 ± .086</td>
<td>0.863 ± .054</td>
<td>0.864 ± .085</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>.294 ± .156</td>
<td>0.455 ± .160</td>
<td>0.723 ± .168</td>
</tr>
<tr>
<td>Muscle</td>
<td>.448 ± .164</td>
<td>0.454 ± .069</td>
<td>0.660 ± .143</td>
</tr>
<tr>
<td>Brain</td>
<td>.677 ± .255</td>
<td>0.553 ± .222</td>
<td>1.670 ± .357 **</td>
</tr>
<tr>
<td>Testicle</td>
<td>.145 ± .056</td>
<td>0.170 ± .070</td>
<td>0.584 ± .106 **</td>
</tr>
</tbody>
</table>

* p-value < 0.05, MDA level significantly different than control  
** p-value < 0.05, MDA level significantly different than control and dietary restricted
significantly increased oxidative stress in the brain, heart, and testes. The anticipated increase in oxidative stress levels in most tissues resulting from simulated microgravity was possibly masked in the brain, heart, and testes by the dietary restriction, which appeared to have a protective effect. The percent reduction in dietary intake needs to be increased for hindlimb-suspended animals since the two-week duration of simulated microgravity has, in previous studies, resulted in an increase in stress levels in these same tissues.

Acknowledgements
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References