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Effects of Dietary Soy Protein on Hematological and Tissue Oxidant/Anti-Oxidant Levels in Rats Exposed to Simulated Microgravity

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Abstract

Effects of a soy protein diet on oxidant and anti-oxidant levels in selected rodent tissues were investigated under simulated microgravity.

Methods: Adult Male Rats were maintained either on a regular Purina Rat chow or on a 9.6% soy protein diet and allowed to remain in either non-suspended (control) or hind-limb-suspended (HLS) state via their tails at a 30 degree angle for 14 days. Body weights, food and water intakes were monitored daily during the entire study period. Hematological parameters, oxidant and. antioxidant levels in the brain, liver and pancreatic tissues were measured.

Results: At term, the body weights of the hind limb suspended animals were found significantly decreased when compared with the non-suspended controls, $p<0.05$. There was no significant change in blood hematocrit and hemoglobin levels. After HLS, malondialdehyde (MDA) levels of brain tissues were marginally increased in the Purina chow group as compared to a significant increase in the soy suspended group ($p<0.05$). Similar observations were noted with liver and pancreatic tissues. In response to HLS, glutathione levels in brain and pancreatic tissues were increased in the Purina chow group ($p<0.05$) while in soy group, glutathione (GT) levels in both of these tissues were found relatively lower. Superoxide dismutase (SOD) levels were increased in all three tissues after HLS regardless of the dietary conditions.

Conclusions: Data suggest that HLS induced a differential tissue specific oxidative response regardless of the dietary differences. Dietary soy protein lowered the oxidant levels under basal state but their effects were not sustained under HLS conditions significantly.

Keywords: Hind Limb Unloading (HLU); Oxidative stress; Dietary soy protein; Simulated microgravity; Oxidant/anti-oxidant levels

Introduction

It is well known that the Imbalance in the oxidant-antioxidant defense system occurs due to the severity of the oxidative stress and it has been documented during space flight and also in aging [1,2]. Oxidative stress during aging is attributed to a generalized increase in free radical production causing a decrease in antioxidant activity**.** The physiologic alterations caused by oxidative stress can be monitored by measurements of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GT) levels. The objective of the current study is to examine the induced oxidative parameters in this rodent model of simulated microgravity that are maintained under two natural dietary conditions for a period of two weeks.

MDA is a known biomarker for oxidative stress [3-5]; it is produced by the decomposition of polyunsaturated fatty acids, and is an end-product of lipid peroxidation. Oxidative stress induces an increase in oxidation of membrane fatty acid moieties leading to production of unstable lipid peroxides which tend to degrade rapidly into products such as malondialdehyde [6]. The formation of these aldehydes contributes to the mechanisms of oxidant-induced injury. Superoxide dismutase (SOD) levels were known to out-compete the damaging reactions of superoxide, thus protecting the cell from superoxide toxicity [7] and it was measured to determine whether its activation is triggered in concert with lipid peroxidation.

Glutathione levels provide natural defense mechanism of cell's oxidative response for it is known that cell's degree of induction of its glutathione antioxidant system may be expected to vary with cell's induction of oxidative capacity resulting from induced HLS and it was measured in this study to examine its role in this mechanism. Reduced glutathione content in the tissues is also an indicator for oxidative stress [7] for it plays a key role in the detoxification of cells by reacting with hydrogen peroxide and organic peroxides.

In this study we evaluated the influence of hind limb suspension on tissue oxidative stress in brain, liver and pancreas of rats. In addition, use of an antioxidant such as dietary soy was conceived with an expected reversal of the membrane peroxidation (and possibly other characteristic changes) presumably by the suppression of MDA formation, and activation of superoxide dismutase, and glutathione levels. The known dietary antioxidants found in the soybean are isoflavones, genistein and diadzein. Hence, soy diet might serve as an effective dietary countermeasure for HLU-induced oxidative stress for it is known that oxidant stress is counterbalanced by activation of endogenous antioxidant levels [7-11].

In earlier studies from our laboratory, a NASA validated rat hind-limb-unloaded (HLU) model of exposure to microgravity was used [12,13]. In this study, hindlimb unloading (HLU) and its effects have been monitored by measurements of malondialdehyde, superoxide dismutase, and glutathione levels in homogenates of selected tissues.

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Materials and Methods

The protocol and procedures used were in accordance with the Guiding Principles in the Care and Use of Animals of the American Physiological Society, and were approved by the University of Arkansas for Medical Sciences (UAMS) Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (n=24) were randomly assigned to 4 groups: those on a regular Purina rat chow diet containing 1.5% soy protein (n=12) or those on a 9.6% soy meal (4.8% soy protein) diet (n=12). Two of the above groups were hind limb unloaded (HLU) by tail suspension (n=12) for 14 days. At term, the animals were sacrificed, blood samples were analyzed for hematocrit, and hemoglobin levels before and after suspension (unloading). Brain, liver and pancreas tissues were harvested, homogenized with phosphate buffer and were analyzed for measurements of malondialdehyde, (MDA), superoxide dismutase (SOD) and total glutathione (GSH and GS-SG).

All animals were acclimated for 1 week prior to the study. Body weights of all animals were monitored on the first day of arrival and again on day seven (the first day of the experiment). Subsequently, the body weights, food, and water intakes were monitored on a daily basis until termination of the experiment. The suspended animals were housed individually in Plexiglas chambers (10×19.5×21 inches) and maintained on 12:12 light-dark cycles. They were allowed free access to tap water and diet.

Hindlimb unloading (HLU) was achieved usinga modification of the Morey-Holton tail-suspension model [14]. HLU was accomplished with a tail harness constructed by looping a 0.5×10-inch Skin-Trac (Zimmer, Inc., Charlotte, NC) orthopedic foam strip around a pulley that can 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 of 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 suspension of the animals at a 30° angle with 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), exsanguinated and sacrificed by decapitation. Brain, liver and pancreas, 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 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. [6] using the LPO-586 method (Bioxytech LPO-586, R&D Systems, Minneapolis, MN 55413). Harvested tissues were washed in ice cold NaCl solution (9gm/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]. Since 10 µL of 0.5 M BHT per ml of homogenate was 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, 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 [6].

Glutathione assay

The glutathione assay reported earlier by Baker et al. [15] was used for our assay. The concentrations of GSSG level in samples were analyzed using a Glutathione Assay Kit from Cayman Chemical Co, Ann Arbor, MI [catalogue no. 703002]. Thekit utilizes enzymatic recycling method using glutathione reductase for the quantification of GSH. The GSH produced from the interaction of sulfhydryl group of GSH with DTNB (5, 5'-dithiobis-2-nitrobenzoic acid) is oxidized to the disulfide dimer GSSG. GSSG is then reduced to GSH by glutathione reductase. The net absorbance at 405 nm yields a linear function of GSSG ranging from 0 to 20 µM. The detection limit for GSSG is 0.1 µM, and these values were extrapolated based on the standard curve provided with the assay kit.

SOD assay

The superoxide dismutase assay (SOD) was determined by the method reported by Maier and Chan [16]. The concentration of superoxide dismutase (SOD) was analyzed using a Superoxide Dismutase Assay kit from Cayman Chemical Co, Ann Arbor, MI [catalogue no. 706002]. The superoxide radical agents formed by the interaction of xanthine oxidase with hypoxanthine are detected by tetrazolium. One unit of SOD detected is equivalent to the amount of enzyme needed to exhibit 50% dismutase of superoxide radical. The net absorbance at 450 nm yields a linear function of SOD ranging from 0 to 0.30 U/ml. The detection limit for SOD is 0.1 U/ml, and these values were extrapolated based on the standard curve provided with the assay kit.

Protein assay

Protein concentrations were measured using the Biuret Method (Bio-Rad Laboratories) described by Bradford [17].

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

Body weights of HLS rats were decreased significantly ($p<0.05$) when compared to their control rats (Table 1). The body weights did not change with diet, and weights of HLS rats remained lower than their control ($p<0.05$) for the duration of the study. Food intake was significantly decreased in the HLS rats when compared to their respective control group (data not shown). Water intake was greatest in the control group and was significantly decreased in the HLS groups. Hematological parameters were not different between control and HLS groups or between diet groups (Table 1).

MDA levels in the brain tissues of the control soy group are lower than in the control Purina chow group, ($P<0.05$) (Table 2). However, in the HLS groups these levels were higher for both diets, but was found significantly different only between control soy and soy HLS groups ($p<0.05$).

Glutathione (GT) levels in the brain tissues of control soy groups are significantly higher than in the control Purina chow groups. Upon suspension glutathione levels significantly increased in Purina chow group. In soy HLS group the levels were significantly lower as compared to their corresponding control group, (P<0.05) (Table 2).

Superoxide dismutase (SOD) levels in the brain tissues of both dietary groups were significantly increased in their corresponding HLS group (p<0.05).

Malondialdehyde (MDA) levels in the liver tissues of the control soy groups are lower than the control Purina chow groups (Table 3). In the Purina HLS groups these levels were not changed; however, the MDA levels were significantly higher in soy HLS group (P<0.05) as compared to their corresponding soy control group.

Glutathione (GT) levels in the liver tissues of soy control groups are significantly lower than the control Purina control groups. Upon suspension these levels are higher in both Purina chow and in soy diet group. However, as compared to Purina chow group the GT levels remained lower in the soy diet group, p<0.05.

Superoxide dismutase (SOD) levels in the liver tissues were not significantly different between the control and the HLS group but in the HLS groups, they were elevated in both dietary groups as compared to their corresponding control groups (P<0.05) in these tissues (Table 3).

MDA levels in the pancreatic tissues of the control soy groups are lower than in the control Purina chow groups (Table 4). However, in the HLS groups these levels were higher for soy diet group as compared to their corresponding control group.

Glutathione (GT) levels in the pancreatic tissues of control soy groups are higher than in the control Purina chow groups, (P<0.05) (Table 4). Upon suspension these levels were significantly higher in control Purina chow group but significantly lower in soy diet group (P<0.05).

Superoxide dismutase (SOD) levels in the pancreatic tissues for both dietary groups were not significantly different from each other but in the HLS groups, these levels were higher as compared to their corresponding controls However, the values are not statistically significant (Table 4).

Discussion

Data from our study suggest that HLS induced a differential tissue specific oxidative response regardless of the dietary differences. Dietary soy protein lowered the oxidant levels under basal state but their effects were not sustained significantly under HLS conditions. This may be attributed due to the fact that 9.6% soy meal used in this study contained approximately 47% of soy protein according to the supplier's brochure. This concentration of soy protein used by the animals as a dietary source for a period of two weeks may not be sufficient to induce the desirable effect.

Cellular membrane lipid peroxidation results from an imbalance in the cell's oxidant-antioxidant defense system leading to the condition where antioxidant defenses are overwhelmed by oxidant production. During space flight, oxidative stress is attributed to the loss of antioxidant protein defenses secondary to an in-flight reductive modeling of skeletal muscle from the decreased workload

		Control		HLS		
	Diet	**Mean**	**SEM**	**Mean**	**SEM**	**P Value**
Final Body Weight (g)	Purina Soy	355 340	30 35	290 300	5 20	<0.01* <0.029*
Hemoglobin (g/dl)	Purina Soy	11.8 11.5	1.1 1.5	12.8 10.4	1.7 1.4	0.47 0.26
Hematocrit (grams)	Purina Soy	40 43	2 2.5	39 37.5	4.8 7	0.86 0.15

*; p<0.05 between control and HLS group

Table 1: Terminal body weights and hematological measurements for control and suspended animals on the two diets

		Control		HLS		
	Diet	**Mean**	**SEM**	**Mean**	**SEM**	**P Value**
Brain MDA(uM/mg of protein)	Purina Soy	0.37 0.30	0.04 0.01	0.385 0.374	0.075 0.076	0.69 0.003*
Brain GT (uM/mg tissue)	Purina Soy	45.0 92.0	23 17	80.0 75	22 17	0.05* 0.015*
Brain SOD (U/ml)	Purina Soy	0.36 0.36	0.07 0.09	0.48 0.47	0.09 0.04	0.02* 0.02*

*; p<0.05 between control and HLS group

Table 2: Oxidant/anti-oxidant parameters of the brain tissue.

		Control		HLS		
	Diet	**Mean**	**SEM**	**Mean**	**SEM**	**P Value**
Liver MDA (uM/mg of protein)	Purina Soy	0.31 0.26	0.01 0.065	0.295 0.345	0.01 0.05	0.71 0.01*
Liver GT (uM/mg tissue)	Purina Soy	480 310**	130 50	530 330**	70 100	0.44 0.68
Liver SOD (U/ml)	Purina Soy	0.35 0.39	0.09 0.05	0.41 0.45	0.05 0.11	0.22 0.20

*; p<0.05 between control and HLS group; ** p<0.05 between Purina control and soy control group

Table 3: Oxidant/anti-oxidant parameters of the liver tissue.

		Control		HLS		
	Diet	**Mean**	**SEM**	**Mean**	**SEM**	**P Value**
Pancreas MDA (uM/mg of protein)	Purina Soy	0.3 0.245	0.085 0.045	0.26 0.29	0.115 0.05	0.49 0.14
Pancreas Glutathione (uM/mg tissue)	Purina Soy	1.3 2.5**	0.4 0.75	2.25 1.2	2.0 0.55	0.8 0.04*
Pancreas SOD (U/ml)	Purina Soy	0.41 0.31	0.06 0.12	0.47 0.34	0.08 0.07	0.14 0.65

*; p<0.05 between control and HLS group; **p<0.05 between Purina control and soy control group

Table 4: Oxidant/anti-oxidant parameters of the pancreatic tissue.

on the antigravity muscles [1,18]. The 9.6% soy meal diet used in this study reduced oxidant levels in all control tissues studied suggesting a positive role in reducing oxygen radicals under basal conditions. The soy diet, however, did not significantly affect oxidant levels in HLU animals, suggesting the dosage of soy protein may need to be increased to produce effects during stressful stimuli.

Recent studies from our laboratory using the NASA validated model of exposure to simulated spaceflight have demonstrated that when animals are suspended by their hind limbs, a global oxidative stress to all harvested organs is produced [19]. The current study utilizes NASA's hind limb unloaded (HLU) rat model of exposure to microgravity to monitor whole-body physical and cellular, hematological and oxidative stress changes in harvested brain, liver and pancreatic tissue homogenates. Values were compared between loaded (controls) and unloaded (suspended) tissues obtained from similar organs harvested from animals on a regular Purina diet and animals on a 9.6% soy diet.

The physical changes, body weights of suspended rats, were decreased significantly ($p<0.05$) when compared to their non-suspended control rats. The body weights did not change with diet, and weights of suspended rats remained lower than control body weights for the duration of the study ($p<0.05$). The decreased body weight in the suspended animals was due to the decreased food and water intake in the suspended animals. This data is consistent with our results of previous experiments showing that the decrease in body weight of suspended animals occurs only during the initial two-to-three days of suspension [20]. Following this early interval of adjustment to the suspended state, suspended rats gain weight at a rate equivalent to the rate of weight gain of control rats. This agrees with literature showing that, unlike humans, rats are able to maintain nutritional intake during spaceflight [21]. The hematological values (hematocrit, and hemoglobin) were generally lower in the HLU groups on the soy diet, but not significantly different from non-suspended control values on either diet. This was not expected in the light of the anemia attributed to prolonged spaceflight in both man and animals [22]. Possibly, the duration of suspension needs to be extended to show any signs of anemia. Thus HLU alters metabolic parameters as reflected by reduction in body weight, but does not have a major effect on hematologic parameters during this period of the study.

One major function of oxygen metabolism is its interaction with polyunsaturated fatty acids in the process described as lipid peroxidation. When lipid peroxidation occurs in biological membranes there may be gross disturbances in structure/function changes leading to injury or possible death of the affected cells [23]. Studies conducted on astronauts and cosmonauts to examine lipid peroxidation [24-27] and on rats returning from space [28-31] have produced conflicting results. Thus it is important to reexamine the oxidant/antioxidant status under stress in animals exposed to simulated microgravity.

Tissue malondialdehyde (MDA) levels are characteristically used to monitor cellular stress as indicators of oxidation of membrane fatty acids. In this study we measured MDA levels in harvested tissues acclimatized to a normal Purina rat chow diet with 1.5% soy protein, or to a 9.6% soy meal diet with 4.8% soy protein, for seven days prior to suspension. The soy chow was used to measure the effectiveness of dietary soy flavonoid proteins as countermeasures for the oxidant-antioxidant defense system. Dietary flavonoids found in soy exhibit powerful antioxidant activities [8,9] in lipid–aqueous systems, and this effect is thought to be due to their ability to increase tissue glutathione (GSH) and superoxide dismutase (SOD) levels which catalyze the dismutation of superoxide into oxygen and hydrogen peroxide [10]. It has long been known [8] that the antioxidative protein compounds in soy, genistein and diadzein, may be responsible for the relatively low rates of heart disease in Asian women and vegetarians, and they are commonly used as a dietary source throughout the world [9]. Total MDA and glutathione (GSH plus GS-SH) and superoxide dismutase (SOD) levels were measured in this study in harvested brain, liver and pancreas.

Superoxide dismutase (SOD) levels were measured in harvested tissues because its toxic product, hydrogen peroxide (H_2O_2), is removed by glutathione peroxidases utilizing the reducing power of GSH [7]. The product of SOD is formedwhen two molecules of GSH are oxidized to form the disulfide-bonded compound, GS-SG, in the reduction of a molecule of hydrogen peroxide. GSH may cooperate with SOD in helping to remove free radicals *in vivo*. Superoxide is one of the main reactive oxygen species in the cell, and, as such serves the role of a key antioxidant. The mutations of this enzyme may cause various pathologies. SOD's generally have high antioxidant capacity [7].

Oxidant (MDA) levels were generally lower in all tissues in the non-suspended groups, but tended to increase in the hind limb unloaded animals in all tissues. This increase identified the degree of cellular "oxidative stress" resulting from simulated weightlessness (suspension, un-loading). The measurement of antioxidant levels appears to be tissue specific, but parallels the oxidant response in HLU animals. Antioxidant (SOD and GSH) levels did not vary significantly between diets, but were generally higher in the suspended animals, regardless of diet. This trend only differed in the brain and pancreas where GSH levels were lower in the suspended animals regardless of diet. This could imply that brain and pancreas were able to overcome the oxidative stress of simulated weightlessness by using only their glutathione antioxidant systems.

In earlier studies in rats we showed that hindlimb-induced oxidative stress was reduced or eliminated by the antioxidants aminoguanidine and L-carnitine supplied in the drinking water [12,13]. These anti-oxidants have been shown to reduce oxidative stress resulting from unloading, L-carnitine, via the enhancement of mitochondrial antioxidant activity and aminoguanidine, via the inhibition of nitric oxide production. The 9.6% soy meal diet used in the current study

reduced oxidant levels in all control tissues studied suggesting a positive role in reducing oxygen radicals under basal conditions. The soy diet, however, did not significantly affect oxidant levels in HLU animals, suggesting the dosage of soy protein may need to be increased to produce effects during stressful stimuli. The measurement of antioxidant levels appears to be tissue specific, but parallels the oxidant response in HLU animals. Antioxidant (SOD and GSH) levels did not vary significantly between diets, but were generally higher in the suspended animals, regardless of diet. This trend only differed in the brain and pancreas where GSH levels were lower in the suspended animals regardless of diet. This could imply that brain and pancreas were able to overcome the oxidative stress of simulated weightlessness by using only their glutathione antioxidant systems.

Conclusions

Our data suggest that hind-limb unloading induced a differential tissue specific oxidative response as determined by measurements of MDA, SOD and GSH levels. Soy protein (9.6%) in the diet lowered basal peroxide levels, but did not affect changes in peroxide levels induced by unloading.

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