Effects of Various Dietary Protein Contents on Vitamin A Status of Rats Exposed to Prolonged Immobilization through Suspension

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(Received September 6, 1990)

Summary The investigation was carried out to clarify the effects of various dietary protein contents on vitamin A status of rats exposed to prolonged immobilization through suspension. A rat wearing a special jacket to which metal chains were attached, was suspended for 10 days as an analogy of simulated weightlessness. Five groups of suspended rats were fed on the diets containing various amounts of casein (5, 10, 20, 40 and 60, w/w%), while control group received the 20% casein diet. Through suspending animals, a decrease in body weight gain and increase in adrenal weights occurred. Serum albumin concentration of the suspended rats fed on the 10, 20, 40 and 60% diets were the same as that of the control rats. The suspended rats showed lowered serum retinol concentrations and elevated hepatic retinyl palmitate contents without noticeable differences between the diets. The hepatic retinol levels were not clearly affected. In the suspended rats, testicular levels of retinyl palmitate and retinol significantly decreased as compared with the control. These parameters' alterations did not relate to serum albumin concentration and were independent of dietary protein levels. The results suggest that stress state may cause suppression of releasing hepatic vitamin A, resulting in a lowered serum retinol concentration, being independent of nutritional status of protein.

Key Words stress, prolonged immobilization, retinol, retinyl palmitate, dietary protein levels

Exposing of animals and humans to various kinds of stressful stimuli such as fevers (1), surgical operation (2), burn injury (3) and chronic immobilization (4) has been linked with lowered serum levels of retinol. A decrease in special retinol-transport protein in plasma, i.e., retinol-binding protein (RBP), was also
observed in patients with infections (5), with burn injuries (6), and with stress arising from multiple trauma (7). However, mechanism(s) responsible for the stress-related alteration of vitamin A state remain to be elucidated.

Space flight is assumed to stimulate environmental stress which may cause various changes in hormone secretion (8). These hormonal changes may lead to alterations in nutrition and metabolism in the body. Based on the information obtained from space missions, space flight was accompanied by alterations of various mineral and nitrogen balance (9). Furthermore, it was reported that the B vitamins and vitamin C concentrations in serum were decreased in space crewmen (10). However no information is available about vitamin A status in space crewmen.

Taking this information into consideration, the questions arise as to whether space flight-associated weightlessness may be accompanied by stress and might cause changes in the vitamin A status of humans as well as animals. It is not clear as to whether space flight-related stress conditions would result in increased demands on the micronutrients, especially vitamin A. The previous study (11) demonstrated that prolonged immobilization-induced stress as an analogy of simulated weightlessness by suspending animals exerted changes in the vitamin A status of rats. The most significant results obtained from the previous study included findings that hepatic retinyl ester contents were elevated in the suspended rats, possibly resulting in a low level of serum retinol.

A very high correlation between plasma retinol and RBP levels for severe malnutrition was observed in human (12). The low serum retinol vitamin A in the patients with kwashiorkor largely reflected a functional impairment in the hepatic release of vitamin A due to defective hepatic production of RBP (12). Acute and chronic liver dysfunctions bring about lowering of serum levels of retinol and its transport protein, RBP (12).

Therefore, we could not rule out the possibility that the prolonged immobilization-induced stress might change nutritional status of protein, followed by altering vitamin A status, e.g., accumulation of hepatic retinyl palmitate and a low serum retinol concentration (11). This study was designed to clarify the effects of various amounts of dietary protein intake on the vitamin A status in rats exposed to prolonged immobilization-induced stress.

MATERIALS AND METHODS

Animals. Male Wistar-strain rats weighing from 170 to 210 g (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were housed in individual wire cages in a temperature- and humidity-controlled room (23°C and 50% relative humidity). Five rats were used in the experimental subgroups. To create a simulated weightlessness condition, the animals of the suspension group were treated as described previously (13). Briefly, a rat was fitted with a special jacket to which metal chains were attached. The rat was suspended by hanging up the
DIETARY PROTEIN LEVEL AND VITAMIN A STATUS IN STRESS CONDITION 445

Table 1. Composition of the experimental diet.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Casein</th>
<th>5%</th>
<th>10%</th>
<th>20%</th>
<th>40%</th>
<th>60%</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>g/100 g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>56.6</td>
<td>52.6</td>
<td>46.6</td>
<td>32.6</td>
<td>19.6</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>27.3</td>
<td>26.3</td>
<td>22.3</td>
<td>16.3</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>Corn oil</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Mineral mix.</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Vitamin mix.</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td></td>
</tr>
</tbody>
</table>


metal chains to the four corners of a wire cage. The animals were able to touch a food vessel and feed under the suspension condition.

An examination was carried out to clarify whether dietary protein intake might affect vitamin A status of the suspended rats. Suspended rats were fed on diets containing various levels of casein (5, 10, 20, 40 and 60 w/w%) freely for 10 days. The control rats received 20% casein diet. The details of these synthetic diet compositions are shown in Table 1. All animals received deionized water freely.

After the experimental period, the animals were decapitated and blood samples were collected using a funnel. The blood was separated to serum. Livers and testes were removed immediately after dissection, rinsed with cold saline, then kept at −80°C until used for analyses.

**Tissue preparation.** Approximately one g of minced testes and livers was homogenized in 9 volumes of the homogenate buffer containing 0.25 M sucrose, 50 mM Tris-HCl (pH 7.5), 25 mM MgCl₂, 16 mM EDTA and 40 mM L-ascorbic acid using Teflon pestle. The homogenate was kept at −80°C for subsequent analyses.

**Extraction and assay of retinol and retinyl palmitate.** Retinol and retinyl palmitate were determined by HPLC analysis. Standard curves were constructed for peak area ratio vs. weight ratio of retinyl acetate and retinol or retinyl palmitate (14). Standard preparations of retinyl acetate and retinyl palmitate were purified from commercial compounds (purchased from Sigma Chemical Co., St. Louis, MO) by chromatography on columns of neutral aluminum oxide weakened with water (5%) (15).

Aliquots (1–2 ml) of the homogenate were gently mixed in glass-capped brown tubes with 1 volume of ethanol containing a known amount of the purified retinyl acetate as internal standard. The brown glass tubes were used after heat treatment at 150°C for 2 h to eliminate fluorescence contamination. The samples were then
extracted with 5 volumes of n-hexane containing butylated hydroxytoluene (BHT) (2 mg/dl) using a vortex mixer for 2 min. After centrifugation at 400×g for 10 min at 4°C, the bulk of the hexane extract sample was withdrawn and evaporated under nitrogen. The residue was dissolved in 100 μl of methanol. A portion of the extracted sample (20 μl) was analyzed by a reverse phase HPLC using a liquid chromatography system (Shimadzu LC-6A) on a μBondapak C18 column (10 nm particle, 3.9 mm i.d. × 25 cm) using 95% methanol/5% water at a flow rate of 1 ml/min. Fluorescence of the retinoids in the eluates was determined using a spectrofluorophotometer (Shimadzu RF-530) with excitation at 325 nm and emission at 460 nm.

Other determinations. The analyses of pure standard solutions for HPLC and the extracted retinol and retinyl palmitate from the specimen were performed by a spectrophotometer (Shimadzu UV-265 FS) and fluorophotometer (Hitachi 650-10S). Working standards were checked by determination of the spectral scan of absorption and fluorescence before each assay.

DNA was assayed by the diphenylamine method using calf thymus DNA as standard (16).

Statistics. All results were subjected to one-way analysis of variance. Differences in mean values between groups were tested using Duncan's multiple range test. Significant differences were justified at p < 0.05 (17).

RESULTS

Effects of stress induced by suspending animals on food intake and body weight

Each group of the suspended rats showed a lower food intake compared with the control rats fed the 20% protein diet (average food intake 175±4 g/10 days). The food intake of suspended rats was 120±7 g for the 5% casein group, 124±5 g for the 10% casein group, 114±3 g for the 20% casein group, 105±4 g for the 40% casein group and 91±3 g for the 60% casein group. The weight gains during a 10-day period of the suspended rat groups were lower than that of the control. The weight gain for the control was 20±3 g/10 days, whereas the suspended rats showed −5±2 g for the 5% casein group, 7±3 g for the 10% casein group, −6±2 g for the 20% group, −4±1 g for the 40% casein group and −8±3 g for the 60% casein group.

Effect of various dietary protein contents on serum concentration of retinol and albumin, and adrenal weight of rats exposed to prolonged immobilization through suspension

Table 2 shows that serum retinol levels in suspended rats were also lower than in the control group. However, each serum retinol level among the groups of suspended rats was almost the same. Thus the dietary protein contents were not related to the serum retinol levels of suspended rats. Serum albumin concentration of the suspended rats fed on the 10, 20, 40 and 60% casein diets are the same as that
The adrenal weights were determined to evaluate whether suspended animals were in a stress condition (18). Table 2 shows the adrenal weights. The suspended rats showed greater weight of the adrenals than that of the nonsuspended control, probably responding to a stress condition. When the adrenal weights were expressed based on unit 100 g body weight, suspended animals again exhibited a significantly elevated weight. All groups of suspended rats showed an increase of the adrenal weights as compared to the control. This increase in adrenal weight related to the declined serum retinol concentration brought by the suspension of the animals.

Effects of various dietary protein contents on vitamin A levels in liver and testis of rats exposed to prolonged immobilization through suspension

The dietary protein levels did not affect the hepatic retinol and retinyl palmitate in suspended rats (Fig. 1). There were no significant differences of these parameters in the liver among the five suspended rat groups (from the 5 to 60% casein groups). When compared to the control group, the levels of hepatic retinyl palmitate in all the groups of suspended rats were 2 times greater. In contrast, the hepatic retinol levels in every group of suspended rats fed the diets containing various amounts of dietary protein did not differ from that in the control group, except for the 40% casein group which showed a slight increase in the hepatic retinol content as compared to the control.

The liver weight in the 5 groups of the suspended rats decreased compared to nonsuspended animals of the control group (the control group, 9.1 ± 0.4 g; the 5% casein group, 5.4 ± 0.3 g; the 10% casein group, 5.2 ± 0.3 g; the 20% casein group, 4.6 ± 0.38 g; the 40% casein group, 4.5 ± 0.4 g; the 60% casein group, 4.8 ± 0.3 g). The hepatic retinyl palmitate contents in the whole liver (mg/liver) were: the

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum retinol (µg/dl)</th>
<th>Albumin (mg/ml)</th>
<th>Adrenal weight (mg/100 g body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (5)</td>
<td>84.5 ± 3.1a</td>
<td>4.1 ± 0.1a</td>
<td>15 ± 1a</td>
</tr>
<tr>
<td>Suspension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% casein (5)</td>
<td>45.2 ± 5.0b</td>
<td>3.5 ± 0.1b</td>
<td>25 ± 2b</td>
</tr>
<tr>
<td>10% casein (5)</td>
<td>49.2 ± 6.8b</td>
<td>3.9 ± 0.2b</td>
<td>22 ± 1b</td>
</tr>
<tr>
<td>20% casein (4)</td>
<td>41.4 ± 1.3b</td>
<td>4.1 ± 0.1a</td>
<td>28 ± 1b</td>
</tr>
<tr>
<td>40% casein (4)</td>
<td>41.3 ± 1.6b</td>
<td>4.3 ± 0.1a</td>
<td>24 ± 2b</td>
</tr>
<tr>
<td>60% casein (5)</td>
<td>41.3 ± 4.0b</td>
<td>4.2 ± 0.2a</td>
<td>28 ± 1b</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. Number of rats given in parentheses. Values not sharing common superscript letter are significantly different at p < 0.05 as assessed by Duncan's multiple range test.
Fig. 1. Effects of various dietary protein contents on retinol and retinyl palmitate levels in liver and testis of rats exposed to prolonged immobilization through suspension. Each bar represents the mean±SEM. Suspended animals received the diets containing various amounts of casein (5, 10, 20, 40 and 60 w/w%) as defined in Table 1. The control group received the 20% casein diet. Values sharing a symbol indicate significant difference from the control group at \( p < 0.05 \) (*) or \( p < 0.01 \) (**) as determined by Duncan’s multiple range test. There were no differences among the suspended rat groups.

<table>
<thead>
<tr>
<th>Protein Content (w/w%)</th>
<th>Liver Retinyl Palmitate (µg/g)</th>
<th>Liver Retinol (µg/g)</th>
<th>Testis Retinyl Palmitate (µg/g)</th>
<th>Testis Retinol (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.80±0.02</td>
<td>3.0±0.2</td>
<td>0.3±0.01</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td>5% casein</td>
<td>2.14±0.22</td>
<td>3.5±0.3</td>
<td>0.4±0.02</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>10% casein</td>
<td>1.92±0.1</td>
<td>3.2±0.2</td>
<td>0.3±0.01</td>
<td>0.10±0.01</td>
</tr>
<tr>
<td>20% casein</td>
<td>1.87±0.02</td>
<td>2.9±0.1</td>
<td>0.3±0.01</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td>40% casein</td>
<td>1.96±0.16</td>
<td>3.0±0.2</td>
<td>0.4±0.02</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>60% casein</td>
<td>1.66±0.20</td>
<td>3.5±0.3</td>
<td>0.4±0.02</td>
<td>0.11±0.02</td>
</tr>
</tbody>
</table>

Since the food intakes of the suspended rat groups decreased, vitamin A intakes were presumably lower in these groups. However, retinyl palmitate contents in the whole liver were almost the same as that in the control group. This suggests that a release of hepatic vitamin A of the suspended animals might be suppressed by the immobilization.

Testis levels of both retinol or retinyl palmitate were significantly reduced in each group of suspended animals fed on the diets containing various protein contents, when compared with the control animals. However, among the groups of suspended rats, there were no differences in retinol and retinyl palmitate levels in the testes, indicating that the increases in dietary protein levels were ineffective to prevent the reduction of testicular retinyl palmitate storage responding to prolonged stress condition.

DISCUSSION

In this study, we suspended animals in an analogy of weightlessness accompanied by stress condition. Although it is difficult to produce zero gravity in ground-based studies, one can obtain a great deal of information about acclimation of animals and humans to weightlessness by using a variety of simulation or analogies such as hypokinesia, prolonged bed or chair rest, immobilization and others (19). Although the suspension of animals cannot be strictly called a simulation of zero gravity, because of the presence of gravity, the suspended state of animals brought about similar physiological changes to those observed in space. For example, when rats were suspended for 10 days with our suspension apparatus, this resulted in the muscle atrophy (13, 20) and urinary calcium loss (unpublished data). It is well known that space flight causes muscle atrophy and bone demineralization (9). In this point, our suspension model can be useful for the simulation of weightlessness associated with stress.

Our data indicate that the reduction of serum retinol concentration in suspended animals accompanied the increase in adrenal weights (Table 2). This enlargement of adrenal tissue suggested that the suspended animals were in stress condition. This stress condition seems to be associated with the alteration in the vitamin A status which included a low serum retinol concentration and an increase of hepatic retinyl palmitate in this study (Table 2, Fig. 1).

Comprehensive studies have evidenced that serum retinol is transferred in complex form with RBP and transthyretin (TTR), and that serum retinol levels are regulated by the RBP metabolism (12). The serum retinol is known to be influenced by nutritional factors with respect to dietary variety such as energy, protein and vitamin A. Protein depletion status reportedly leads to a low serum retinol concentration, because of disorder of RBP synthesis in liver due to lack of substrates. It was expected that stress induced by suspension of animals may cause enhancement of protein catabolism, resulting in low levels of RBP and serum retinol. However, the data indicate that the dietary protein intake levels were not critical for their serum retinol levels in the suspended rats. There were no differences in the serum retinol levels between the groups of suspended rats fed the diets containing various amounts of casein from 5 to 60% in the diet (Table 2). Besides, the declined serum retinol concentrations of these suspended groups did not relate to the serum albumin concentrations which were the same as that in the control group (Table 2). This finding suggested that RBP-retinol secretion from liver into serum in the suspended rats decreased by different mechanism from that for hepatic albumin secretion. Previous study (21) explored that RBP-retinol secretion process is involved in Golgi apparatus, Golgi-derived secretory vesicles and microtubules, and that this secretion process did not relate with the overall rate of hepatic protein synthesis.

The suppression of releasing hepatic vitamin A which could be ascribed to
declined retinol-RBP complex mobilization into blood, was independent of protein intake levels. Each group of suspended rats fed on various protein levels in the diets, showed considerably higher levels in hepatic retinyl palmitate as compared with the control group, without noticeable differences between the dietary groups of suspended rats. Our previous study revealed that a rise in hepatic retinyl palmitate contents in the suspended rats was also independent of the energy intakes (11). In the previous study (11) in which pair-feeding and force-feeding were carried out, the suspended rats exhibited remarkably elevated hepatic retinyl palmitate levels, even though animals ingested the lower amounts of energy due to the decreased food intake. Furthermore, although the suspended rats consumed the same amounts of energy as that consumed by the normal rats by force-feeding technique, the hepatic retinyl palmitate level increased. Therefore, it suggests a possibility that stress state arising from exposing animals to prolonged immobilization through suspension leads to a rise in hepatic retinyl ester content and to a fall in serum retinol concentration. These parameters’ alterations are probably independent of nutritional changes in protein and energy metabolism. Mechanisms responsible for this suppression of releasing hepatic vitamin A are not clear at present.

Reportedly, stress arising from the zero gravity condition brought about an increase in secretions of the adrenocorticoid and thyroid hormone (T4) (9). These hormonal factors in stress condition appear to be connected to the suppression of releasing hepatic vitamin A. Thyroid hormone reportedly relates to serum retinol and RBP levels. The patients with hyperthyroidism showed decreased serum levels in retinol, RBP and TTR (22).

The level of retinyl palmitate in the peripheral target tissues of vitamin A action such as testes remarkably decreased in each group of the suspended rats exposed to prolonged immobilization-induced stress relative to the control. Nevertheless its levels were independent of protein intake levels varying from low to high in this study (Fig. 1). Altering retinyl palmitate contents in peripheral tissue, therefore, may be the consequence of an increased rate of retinol utilization and/or low uptake of circulating retinol.

Further study is needed to clarify the mechanism(s) which cause the suppression of releasing hepatic vitamin A in rats exposed to prolonged immobilization-induced stress.

This study was supported by the Science and Technology Agency, Institute of Aerospace Technology, Tokyo, and the Ministry of Education, Institute of Space Science, Sagamihara, Kanagawa, Japan (U-9).

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Vol. 37, No. 5, 1991


