INTRODUCTION

Daily exposure to social and psychological stress is an environmental factor that disturbs health, and, hence, is considered to be associated with lifestyle-related diseases such as hyperinsulinemia, hyperglycemia, cardiovascular diseases, obesity and cancer (Ader and Cohen, 1993; McEwen and Stellar, 1993; Raikkonen et al., 1996; Chrousos and Gold, 1998; Chandola et al., 2006; Brunner et al., 2007; Danese et al., 2009). This indicates that humans are simultaneously exposed to social stress and environmental chemicals in the course of their daily life.

We recently investigated the biological responses of mice exposed to social stress comprising isolation with a reduced volume of bedding (2 g/cage) (Miyashita et al., 2006; Nishio et al., 2007; Motoyama et al., 2009). In our system, the control group was the grouped-housing (5 mice/cage) with a standard volume of bedding (40 g/cage). We reported several findings indicating that social isolation stress for 7 days exerts typical stress responses compared with the control, such as adrenal hypertrophy and increased blood corticosterone levels (Miyashita et al., 2006). Additionally, oxidative DNA damage in peripheral blood cells and urinary excretion of biopyrins, oxidative metabolites of bilirubin, were shown to be significantly increased (Miyashita et al., 2006).
We also have analyzed the hepatic gene expression profiles of the mice exposed to social isolation stress for 4 weeks using DNA microarrays. The lipid metabolism-related pathway through the peroxisome proliferator-activated receptor alpha subtype was remarkably down-regulated, and the lipid biosynthesis pathway controlled by sterol regulatory element binding factor 1 was significantly up-regulated without changing body weight compared with the control (Motoyama et al., 2009).

Above all, these results led us to hypothesize that prolonged social isolation stress for more than 4 weeks would activate lipid biosynthesis and enhance energy accumulation, particularly in the liver, and result in excess weight gain and hepatic hypertrophy. Therefore, in this study, we investigated the physiological effects of prolonged social isolation stress on mice. After 13 weeks of social isolation stress, the liver, visceral fat, thymus and adrenal glands were weighed. The impact on hepatic steatosis was investigated by histological analysis. In addition, plasma parameters including corticosterone and adiponectin were determined.

**MATERIALS AND METHODS**

**Animals and housing conditions**

Male C57BL/6J mice (4 weeks old, Japan SLC, Shizuoka, Japan) were housed five mice per cage (338 × 225 × 140 mm) for 10 days. After acclimatization, mice were exposed to social stress comprised of isolation with a reduced volume of bedding (2 g/cage) for 13 weeks in the same size cage, according to the modified method described in our previous report (Miyashita et al., 2006). The control group was continued under the same condition as the acclimatization period with a standard volume of bedding (40 g/cage). Individual cages were placed in a foam plastic box to prevent social contact with other cages. Both groups consisted of fifteen mice. All animals were housed in an air-conditioned room (23 ± 1°C) under a 12-hr dark/light cycle (light on 8:00-20:00) with free access to tap water and purified control diet (3.8 kcal of energy per gram; D12450B, Research Diets, Inc., New Brunswick, NJ, USA). The bedding used was commercial chip made from fir trees. All experimental procedures were performed in accordance with the guidelines of the University of Shizuoka, Japan, for the Care and Use of Laboratory Animals, based on those of the American Association for Laboratory Animal Science. The social isolation employed in this study induces a stress response, as described above, in mice. Hence, mice were checked each day for adverse effects. If extreme symptoms, such as hair loss, appeared, the animal was removed from the experiment. All efforts were made to minimize suffering.

**Sample collection**

At week 13, the mice were fasted for 6 hr and blood samples were taken from the abdominal vein into heparinized Capiject® tubes (Terumo Medical Corporation, Somerset, NJ, USA) under anesthesia using ether. Plasma was obtained by centrifugation (1,200 × g, 10 min), and stored at -80°C until analysis. The liver, thymus, adrenal glands and one part of the visceral fat (mesenteric and peritestis) were weighed. To eliminate the technical differences between the prosectors, the same researchers collected the same organs. The left lateral lobe of the liver was cut into sections about 5 mm thick and fixed in 10% buffered formalin for histological analysis. The sample collections were conducted between 17:00 and 19:00.

**Plasma biochemical analyses**

Six plasma biochemical parameters, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and creatine kinase activities, triglyceride, and free fatty acid levels, were determined using an automated analyzer TBA-120FR (Toshiba Co., Tochigi, Japan) with standards from Wako Chemicals (Osaka, Japan). All standards were used in accordance with the manufacturer’s instructions and within the stated expiration date. Leptin, adiponectin and insulin levels were analyzed using mouse enzyme-linked immunoassays obtained from the Morinaga Institute of Biological Science, Inc. (Kanagawa, Japan), Otsuka Pharmaceutical Co., Ltd (Tokyo, Japan), and Mercodia AB (Uppsala, Sweden), respectively. Plasma corticosterone was determined using at EIA kit (Assay Designs, Inc., MI, USA).

**Histologic analysis**

The livers from mice exposed to social isolation stress for 13 weeks and control mice were fixed in 10% neutral buffered formalin. Specimens were prepared for histopathological examination by embedding in paraffin wax, sectioning and staining with hematoxylin and eosin (HE) and Oil red O stains.

**Statistical analysis**

Data are indicated as mean ± standard error. Statistical analyses were performed using the software program Stat View for Windows (Version 5.0, SAS Institute, Cary, NC, USA). The statistical significance between the control and experimental group was evaluated by independ-
ent t-tests. The results were considered to be significant if the possibility of error was less than 5%.

RESULTS AND DISCUSSION

In this study, we found that the body weights of mice exposed to social isolation stress were increased after 6 weeks of housing, and the body weights at the 13 week time point were significantly higher (> 8.0%) than those of the control (Table 1). The liver and visceral fat weights were significantly increased in the group exposed to social isolation stress (Table 2). On the other hand, there were no differences in the weights of the thymus and adrenal glands. Histopathological examination indicated that the severe fatty change in the mid-zonal area was observed in the liver from the mice exposed to social isolation stress (Fig. 1). In this study, the social stress group was isolated in the same size cage as the grouped-housing control, and additionally the volume of bedding was reduced 20 times less than the control to enhance the feeling of isolation. Therefore, the results obtained in this study were compared between such two extremely different environments for mice, but, given these physiological changes, it is tempting to speculate that the C57BL/6J mice exposed to social isolation stress for 13 continuous weeks could be at an increased risk of hepatic hypertrophy with the fatty change in the hepatocytes.

Essentially, the hypothalamic-pituitary-adrenal (HPA) axis is activated and, consequently, corticosterone is secreted into the blood from the adrenal cortex when exposed to stress (Magarinos et al., 1987; Diorio et al., 1993). Hence, we previously reported that plasma cor-

![Fig. 1](image_url)  
Fig. 1. Light microscopic photographs with HE stain of livers in mice exposed to social isolation stress for 13 weeks and control mice. After acclimatization for 10 days, mice were exposed to social stress comprised of isolation with a reduced volume of bedding (2 g/cage). The controls were group housed (5 mice per cage) with standard volume of bedding (40 g/cage). Liver samples were obtained from both groups, and were stained with HE. Representative images are shown.
Steroid levels were significantly increased around 2 times in the BALB/C mice exposed to the social isolation stress at period of 1 week, and following gradually decreased (Miyashita et al., 2006). Additionally, we also confirmed similar results in C57BL/6J mice (data not shown). However, plasma corticosterone levels in this study were significantly lower than that in the controls after 13 weeks of social isolation stress (Table 3). Armario and the other research groups suggested that a habituation to chronic stress exposure, for example a progressive reduction of the HPA response, were observed when animals are exposed daily to the same stressful event for a few weeks (De Boer et al., 1990; Armario, 2006). Hence, the HPA response under long-term social isolation stress, for example 13 weeks, is probably habituated for daily stress.

Blood adiponectin levels are known to decrease under obese conditions (Hagiwara et al., 2009), and our results agree with this observation (Table 3). Plasma triglyceride levels in the group exposed to social isolation stress were significantly decreased, indicating the possibility of the accumulation of triglycerides in the liver. Plasma free fatty acid levels also were decreased under the social isolation stress. This response might be potentially via inhibition of lipoprotein lipase activity under the exposure to social isolation stress, but we have to continue further experiments to inspect the exact mechanisms. Typical changes were not observed on the other blood parameters (Table 3). Bartolomucci et al. (2009) reported that mice exposed to stress showed decreased locomotor activity during the active phase. In our results, blood creatine kinase activity, which is generally considered to increase in blood after exercise such as running (Vilquin et al., 1998), was significantly lower in the group exposed to social isolation stress (Table 3), indicating the possibility that the locomotion was lower. That is, lower energy consumption under the social isolation stress, could be one of the reasons for the increase in the body, liver and visceral fat weights under the social isolation stress. Furthermore, the social isolation stress stimulated food consump-

Table 1. Body weights and food intakes

<table>
<thead>
<tr>
<th>Weeks</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>21.2 ± 0.2</td>
<td>24.6 ± 0.4</td>
<td>28.1 ± 0.4</td>
<td>31.4 ± 0.8</td>
<td>34.3 ± 0.6</td>
<td>35.2 ± 0.6</td>
</tr>
<tr>
<td>Social isolation stress</td>
<td>20.9 ± 0.3</td>
<td>24.5 ± 0.6</td>
<td>28.4 ± 0.8</td>
<td>33.3 ± 1.0</td>
<td>37.1 ± 0.9</td>
<td>38.0 ± 0.8*</td>
</tr>
<tr>
<td>Food intake (g/mouse/day)1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>2.2 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td>3.0 ± 0.1</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Social isolation stress</td>
<td>-</td>
<td>2.5 ± 0.1*</td>
<td>3.7 ± 0.1*</td>
<td>3.9 ± 0.1*</td>
<td>4.0 ± 0.1*</td>
<td>3.7 ± 0.1*</td>
</tr>
</tbody>
</table>

After acclimatization for 10 days, mice were exposed to social stress comprised of isolation with a reduced volume of bedding (2 g/cage) for 13 weeks. Controls were group-housed (5 mice per cage) with a standard volume of bedding (40 g/cage). Individual food intake is indicated as the data from the last three weeks except for the 13-week time point, which are the data from the last week until fasting. The control group, which was composed of 3 cages (5 mice/cage), was calculated as mean values of each cage (n = 3). Values are means ± standard error (n = 15 except for food intake at control, n = 3). *P < 0.05 vs. Control (t-test).

Table 2. Effect of social isolation stress on organ and gland weights of mice

<table>
<thead>
<tr>
<th>Organ weight</th>
<th>Control</th>
<th>Social isolation stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (g)</td>
<td>1.45 ± 0.05</td>
<td>1.82 ± 0.09*</td>
</tr>
<tr>
<td>(mg/g BW)</td>
<td>39.4 ± 1.1</td>
<td>47.4 ± 1.3*</td>
</tr>
<tr>
<td>Visceral fat</td>
<td>2.95 ± 0.12</td>
<td>3.44 ± 0.17*</td>
</tr>
<tr>
<td>(mg/g BW)</td>
<td>83.2 ± 2.3</td>
<td>90.0 ± 2.5*</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>0.0034 ± 0.0002</td>
<td>0.0035 ± 0.0002</td>
</tr>
<tr>
<td>(mg/g BW)</td>
<td>0.099 ± 0.005</td>
<td>0.093 ± 0.006</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.060 ± 0.003</td>
<td>0.061 ± 0.003</td>
</tr>
<tr>
<td>(mg/g BW)</td>
<td>1.83 ± 0.08</td>
<td>1.59 ± 0.06</td>
</tr>
</tbody>
</table>

After acclimatization for 10 days, mice were exposed to social stress comprised of isolation with a reduced volume of bedding (2 g/cage) for 13 weeks. The controls were group housed (5 mice per cage) with a standard volume of bedding (40 g/cage). Values are means ± standard error (n = 15). *P < 0.05 vs. Control (t-tests.).
tion from the beginning of the experiment, although body weight gains were not changed until 9 weeks of exposure (Table 1). This is another possibility for the increase in the body weights after 13 weeks of social isolation stress. However, we should conduct further study to elucidate these differences in time between increasing food intakes and body weights.

Interestingly, Suhail and co-workers reported the effects of psychological stress on carcinogen-induced toxicity (Suhail et al., 2011). In that study, mice were exposed to different types of severe stress, such as restraint, shaking, swimming and fasting for 15 days before administration of a well-known mutagenic agent, 7,12-dimethylbenz (a) anthracene (DMBA), and sacrificed after 16 weeks of treatment. The results indicated pre-exposure to psychological stress might exacerbate the hepatotoxic and nephrotoxic potential of the carcinogens through enhanced oxidative stress. The results obtained in the current study suggested that chronic exposure to social isolation stress might increase the risk of hepatic hypertrophy with the fatty change in the hepatocytes. The same social isolation stress exposure to mice has been reported to induce oxidative stress, as mentioned above (Miyashita et al., 2006; Nishio et al., 2007). Hence, these physiological changes under chronic stress exposure are regarded to be an important factor when evaluating the chronic effects of environmental chemicals.

In conclusion, it is tempting to speculate that the mice placed under social isolation stress continuously for 13 weeks could be at an increased risk of overweight with accumulation of visceral fat. Concurrently, the liver weights were significantly higher with the formation of lipid vacuolization in the stressed group compared to the control. The reasons of these alterations might be because of decreasing locomotor activity and increasing food intakes.

**ACKNOWLEDGMENTS**

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