Rare sugar prevents MEHP dependent testicular atrophy

Shigeru Suna1 and Masaaki Tokuda2

1Department of Medical Technology, Graduate School of Health Sciences, Kagawa Prefectural University of Health Sciences, 281-1 Hara Mure-cho, Takamatsu-shi, Kagawa 761-0123, Japan
2International Office, Kagawa University
E-mail: suna1029@hi3.enjoy.ne.jp

ABSTRACT
Background: Di-(2-ethylhexyl) phthalate (DEHP), the most widely used plasticizer is a reproductive toxicant and is suspected to be an endocrine disruptor. Mono-(2-ethylhexyl) phthalate (MEHP), the major DEHP metabolite, causes testicular injury through oxidative stress in rodents. Therefore, we are concerned about the adverse effect of DEHP that disrupts reproductive health in humans.

Method: To clarify the quantitative relation between MEHP burden and testicular atrophy, four-week-old and five-week-old (age at start) rats were receiving 1 or 2% (w/w) DEHP diet for two weeks, and plasma and testicular MEHP concentrations of the rats were determined. And also, to clarify the protective potency of D-allulose (D-psicose) against MEHP-induced testicular atrophy, four-week-old rats received a 1% (w/w) DEHP diet and 2% (w/w) D-allulose water for two weeks.

Result: Both of four-week-old rats and five-week-old rats given 2% DEHP diet showed a significant reduction of the relative testicular weight resulted in a severe testicular atrophy. On the other hand, in rats given 1% DEHP diet, only the four-week-old rats showed a significant reduction of the testicular weight. MEHP dependent negative correlations were found between plasma MEHP levels, and relative testicular weights and also found between testicular MEHP levels, and relative testicular weights. Although plasma and testicular MEHP levels in the four-week-old rats and five-week-old rats were similar distribution, four-week-old rats were more susceptible to the testicular toxicity than five-week-old rats. In contrast, in the four-week-old rats treated with 1% DEHP diet and 2% D-allulose water for two weeks, relative testicular weights and histologic observations were similar to those of the control group and were independent of MEHP.

Conclusion: These results show that the testicular atrophy induced by DEHP is age dependent and MEHP dependent, and D-allulose, a rare sugar can prevent testicular atrophy in rats exposed to a high concentration of DEHP in their diet. D-allulose may be useful as a preventive for oxidant testite damage in humans and may also be used in endangered species.

Keywords: DEHP; MEHP burden; testicular atrophy; D-allulose; anti-oxidative effect

1. INTRODUCTION

Di- (2-ethylhexyl) phthalate (DEHP) is the most widely used plasticizer in the manufacture of consumer products, food containers, toys and medical devices [1], and is now ubiquitous global pollutant. DEHP shows reproductive toxicity and is suspected to be an endocrine disruptor [2, 3]. Many studies have shown that mono-(2-ethylhexyl) phthalate (MEHP), the major DEHP metabolite, is an active toxicant which causes testicular toxicity through oxidative stress. However the quantitative relations between testicular toxicity and MEHP burden is not well known.

Although toxicity of DEHP to humans remains unclear, some epidemiological studies suggest an association between phthalates exposure and reproductive toxicity among humans [4-7]. Therefore, it is important to investigate the protective agents against DEHP-induced testicular injuries. Previously, we have shown that D-allulose (D-psicose), one of rare sugars, prevents DEHP-induced testicular atrophy by suppressing the generation of reactive oxygen species (ROS) in the rat testis [8].

Present study shows the quantitative relation between testicular atrophy and MEHP burden induced by dietary exposure, and also shows complete protection by D-allulose against MEHP dependent testicular atrophy.
2. MATERIALS AND METHODS

2.1. Chemicals and Animal Diet
Chemical purity > 97% DEHP was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The CE-2 diets (Clea, Tokyo, Japan) containing DEHP were prepared by Oriental Yeast Company (Chiba, Japan). D-allulose was provided by Kagawa Rare Sugar Research Center (Kagawa, Japan). The chemical purity of D-allulose was found to be >98%. Chemical purity > 90% MEHP was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

2.2. Animals and Ethics
Male Sprague-Dawley rats aged three-week-old purchased from Charles River (Kanagawa, Japan) were housed at the Laboratory Animal Center of Kagawa University. They were acclimated at 22–24 °C and 50–60% relative humidity with a 12-h light/dark cycle. The experiment protocols had the approval by the Kagawa University Animal Committee (protocol no. 132/2007).

2.3. Experimental Design
In the first experiment, four-week-old rats weighing 90-110g and five-week-old rats weighing 150-170g were divided into control and treatment groups consisting of 6 animals. The treatment groups received a 1 or 2% DEHP diet and tap water for two weeks.

In the second experiment, four-week-old rats were divided into control and treatment groups consisting of 12 animals. The treatment groups received a 1%DEHP diet and tap water or 2%D-allulose water for two weeks.

At the end of each experiment, rats were sacrificed by ether anesthesia. The testis was removed and weighed. The left testis was immediately fixed in Bouin's fluid. Blood samples collected from the heart were collected into heparinized tubes and plasma was separated from whole blood by centrifugation at 1500 g. Right testis and plasma were frozen at -40 °C until MEHP measurement.

2.4. Plasma and Testicular MEHP Measurement
The concentrations of MEHP in plasma and testis were measured by high performance liquid chromatography. Analytical procedure and equipment are as reported [8]. The recovery rate of control rat plasma spiked at 100 μg / ml with MEHP was 100.9 ± 4.7% (Mean ± SD, n = 6). The recovery rate of control rat testes spiked at 100 μg / g with MEHP was 96.7 ± 2.5% (mean ± SD, n = 6). The detection limit of MEHP was 0.1 μg / ml or 0.1 μg / g.

2.5. Histological Tissue Sample Preparation
After fixing in Bouin's fluid for 2 days, the testis was washed and dehydrated gradually with ethanol series, and embedded in paraffin wax. Five micrometer sections cut from the central part of the testis were stained with hematoxylin-eosin.

2.6. Statistical Analysis
SPSS 12.0 software package and Excel 2016 were used for statistical analyses and regression analysis. Results were expressed as means ± standard deviations (SD). Statistical analysis was performed by one-way ANOVA test followed by Tukey's postanalysis test for multiple comparisons. p <0.05 was considered as statistically significant.

3. RESULTS

3.1. First experiment
Table 1 shows body and testicular weights, relative testicular weights (as a percentage of body weight), plasma and testicular MEHP levels in four-week-old and five-week-old rats treated with 1 or 2% DEHP diet for two weeks. Both of four-week-old rats and five-week-old rats given 2% DEHP diet showed a significant decrease of body weight gain and a significant reduction of the relative testicular weight resulted in a severe testicular atrophy (Figure 1). On the other hand, in rats given 1% DEHP diet, only the four-week-old rats showed a significant reduction of the testicular weight.

As shown in Figure 2, plasma and testicular MEHP levels of the four-week-old and five-week-old rats were closely correlated. Figure 3 shows the relationship between relative testicular weight and plasma and testicular MEHP levels. MEHP dependent negative correlations were found between plasma MEHP levels and relative testicular weights and also found between testicular MEHP levels and relative testicular weights. Although plasma and testicular MEHP levels in the four-week-old rats and five-week-old rats were similar distribution (Figure 2), four-week-old rats were more susceptible to the testicular toxicity than five-week-old rats (Figure 3).
3.2. Second experiment

Relative testicular weights and plasma MEHP levels of the four-week-old rats treated with 1% (w/w) DEHP diet and 2% (w/w) D-allulose water for two weeks are listed in Table 2 and light micrographs of testicular sections from control and treatment groups are shown in Figure 4. The rats exposed to DEHP alone showed a severe testicular atrophy (Figure 4-(b)). On the other hand, the 2% (w/w) D-allulose water provided marked protection against testicular atrophy, resulting in an almost complete spermatogenesis (Figure 4-(c)).

Figure 5 shows the relationship between plasma MEHP levels and relative testicular weights of the control and treatment groups receiving the diets for two weeks. A significant negative correlation was found between plasma MEHP levels, and relative testicular weights in rats given the DEHP-free diet (Control) and 1% (w/w) DEHP diet. However, in rats given the 1% (w/w) DEHP diet plus 2% (w/w) D-allulose water, there was no correlation and no significant difference of relative testicular weight as compared to control.

4. DISCUSSION

It has been shown that orally administered DEHP is rapidly metabolized to MEHP by lipase in the gastrointestinal tract [9]. Lipase activity shows species differences, but it is detected in mammals, including humans. It has been shown that MEHP generates oxidative stress which disrupts Sertoli cell function resulting in germ cell apoptosis, and it has also been reported that MEHP induces increase in Fas ligand expression by Sertoli cells to initiate the apoptosis of Fas-positive testicular germ cells [11-13]. On the other hand, MEHP-induced oxidative stress can directly injure germ cells and cause spermatocyte apoptosis. [14]. It is well known that the DEHP toxicity on male reproductive organs is more sensitive in juvenile rats than in mature [15, 16]. Our previous studies have shown that the DEHP diets cause a severe testicular atrophy in prepubertal rats [8, 17]. In the present quantitative study in relation to testicular atrophy and MEHP burden, MEHP dependent negative correlations were found between plasma MEHP levels and relative testicular weights and also found between testicular MEHP levels and relative testicular weights. Although each of four-week-old rats and five-week-old rats showed a similar distribution pattern in the relation between plasma and testicular MEHP levels, four-week-old rats were more susceptible to the testicular toxicity than five-week-old rats. These results suggest that the testicular toxicity by DEHP is age dependent and MEHP dependent.

Large scale production of rare sugars [18-20] by enzymatic isomerization of sugars was established at Kagawa University, and some rare sugar studies revealed their antioxidant and anti-apoptotic properties [21-23]. In particular, the scavenging activity of D-allulose (D-psicose) was higher than that of other sugars [24]. When orally administered, D-allulose is partly absorbed in the gastrointestinal tract and excreted into rat urine and feces [25]. These studies suggest that D-allulose can eliminate testicular oxidative stress. The mechanism of apoptosis mediated by oxidative stress has been established [26-28], and studies have shown that antioxidants can suppress or delay apoptosis by acting as scavengers of ROS [29, 30]. Thus, antioxidants can reduce MEHP-induced oxidative stress and ultimately testicular damage [17, 31]. Moreover, our previous study showed that D-allulose prevents DEHP-induced testicular injury by suppressing the generation of reactive oxygen species in the rat testis [8].

In the second experiment, a significant negative correlation was found between plasma MEHP levels and relative testicular weights in four-week-old rats receiving a 1% (w/w) DEHP diet alone for two weeks, suggesting a dose dependent relationship between MEHP and testicular atrophy. But, relative testicular weights and histologic observations were similar to those of the control group and were independent of MEHP levels in four-week-old rats receiving a 1% (w/w) DEHP diet plus 2% (w/w) D-allulose water for two weeks. These results show that 2% D-allulose water almost completely inhibited the testicular atrophy in rats receiving a 1% DEHP diet. On the other hand, the fact that D-allulose may not prevent testicular toxicity at low MEHP concentrations (Figure 5) suggested a higher sensitivity to MEHP toxicity in juvenile rats. D-allulose can prevent testicular atrophy in rats exposed to a high concentration of DEHP in their diet. The radical scavenging potency of D-allulose in the testis appears to be considerably high. The reproductive toxicity of DEHP among humans is less clear. However, D-allulose may be useful as a protective agent against oxidant-mediated testicular injury in humans and may also be used in endangered species.

5. ACKNOWLEDGMENTS

The present study was supported by a Grants-in-Aid for Rare Sugar Research of Kagawa University. The authors report no conflicts of interest in this work.
REFERENCES


Table 1. Body and testicular weights, relative testicular weights (as a percentage of body weight), plasma and testicular MEHP levels in four-week-old and five-week-old rats given 1 or 2% (w/w) DEHP diet for two weeks. ***p < 0.001 as compared to Control.

<table>
<thead>
<tr>
<th>Age at start (Initial body weight (g))</th>
<th>Experimental findings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 1%DEHP 2%DEHP</td>
</tr>
<tr>
<td></td>
<td>n 6 6 6</td>
</tr>
<tr>
<td></td>
<td>Final body weight (g)</td>
</tr>
<tr>
<td>4-week-old (102.9±5.8)</td>
<td>228.9 ± 6.3 218.5 ± 20.8 161.6 ± 17.6***</td>
</tr>
<tr>
<td></td>
<td>Testes (g)</td>
</tr>
<tr>
<td></td>
<td>2.04 ± 0.26 1.00 ± 0.32*** 0.73 ± 0.10***</td>
</tr>
<tr>
<td></td>
<td>Relative testicular weight (%)</td>
</tr>
<tr>
<td></td>
<td>0.89 ± 0.10 0.46 ± 0.13*** 0.45 ± 0.05***</td>
</tr>
<tr>
<td></td>
<td>Plasma MEHP (μg / ml)</td>
</tr>
<tr>
<td></td>
<td>- 23.6 ± 6.7 146.1 ± 38.8</td>
</tr>
<tr>
<td></td>
<td>Testicular MEHP (μg / g)</td>
</tr>
<tr>
<td></td>
<td>- 2.88 ± 1.01 21.05 ± 4.60</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>5-week-old (165.8±4.0)</td>
<td>287.1 ± 11.6 280.3 ± 19.2 243.4 ± 11.6***</td>
</tr>
<tr>
<td></td>
<td>Testes (g)</td>
</tr>
<tr>
<td></td>
<td>2.70 ± 0.14 2.52 ± 0.17 1.18 ± 0.36***</td>
</tr>
<tr>
<td></td>
<td>Relative testicular weight (%)</td>
</tr>
<tr>
<td></td>
<td>0.94 ± 0.05 0.90 ± 0.11 0.48 ± 0.14***</td>
</tr>
<tr>
<td></td>
<td>Plasma MEHP (μg / ml)</td>
</tr>
<tr>
<td></td>
<td>- 24.2 ± 8.7 88.2 ± 32.0</td>
</tr>
<tr>
<td></td>
<td>Testicular MEHP (μg / g)</td>
</tr>
<tr>
<td></td>
<td>- 2.08 ± 1.01 18.27 ± 7.60</td>
</tr>
</tbody>
</table>

Table 2. Relative testicular weights and plasma MEHP levels in the four-week-old rats given 1% (w/w) DEHP diet plus 2 % (w/w) D-allulose water for two weeks. ***p < 0.001 as compared to Control.

<table>
<thead>
<tr>
<th></th>
<th>Control 1%DEHP 1%DEHP+2%Allulose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n 12 12 12</td>
</tr>
<tr>
<td></td>
<td>Relative testicular weight (%)</td>
</tr>
<tr>
<td></td>
<td>0.94 ± 0.10 0.40 ± 0.08*** 0.81 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>Plasma MEHP (μg / ml)</td>
</tr>
<tr>
<td></td>
<td>- 27.8 ± 15.0 27.5 ± 15.7</td>
</tr>
</tbody>
</table>

Figure 1. Light micrographs of testicular seminiferous tubules in control and treated groups (original magnification: 200×). (a) Five-week-old rats given the DEHP-free diet for two weeks. (b) Five-week-old rats given the 2% (w/w) DEHP diet for two weeks.
Figure 2. Correlation between plasma and testicular MEHP levels of the four-week-old and five-week-old rats. Open circle; four-week-old rats ($r = 0.968$). Closed circle; five-week-old rats ($r = 0.939$).

Figure 3. Relationship between plasma MEHP levels and testicular weights of the four-week-old and five-week-old rats given 1 or 2% (w/w) DEHP diet for two weeks. Open circle; four-week-old rats. Closed circle; five-week-old rats. Regression lines and equations are calculated from five-week-old rats.
Figure 4. Light micrographs of testicular seminiferous tubules in control and treated groups (original magnification: 200×). (a) Four-week-old rats given the DEHP-free diet for two weeks. (b) Four-week-old rats given the 1% (w/w) DEHP diet for two weeks. (c) Four-week-old rats given the 1% (w/w) DEHP diet plus 2% (w/w) D-allulose water for two weeks.

Figure 5. Relationship between plasma MEHP levels and testicular weights of control and treatment rats receiving the diets for two weeks. Open circle; four-week-old rats given the DEHP-free diet (Control) or 1% (w/w) DEHP diet. Closed circle; four-week-old rats given the 1% (w/w) DEHP diet plus 2% (w/w) D-allulose water. Regression lines and equations are calculated from the Control group and the 1% (w/w) DEHP diet group.