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Severe abnormalities in the reproductive organs of mice caused by chemical substances contained in heavy oil

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ABSTRACT — It is well known that heavy oil pollution results in various negative impacts on the marine environment. Although there is a low possibility of direct exposure to heavy oil, the chemical substances contained in heavy oil may be released into the environment and accumulated by marine organisms which in turn can be taken by humans via the food chain. In this study, we examined the biological risk of heavy oil extract using the common mouse, whose genetic backgrounds and immune system are well known and relatively homologous to humans. Water-soluble fraction (WSF) was extracted from heavy oil with water and the extract orally administrated to female or male mice for 7 days. In the WSF administrated group, cystoma-like formation was observed in the ovary in approximately 80% of female mice. On the other hand, we found that the prostate gland size in male mice was markedly reduced in comparison with male control mice. Continuous administration of WSF for 28 days resulted in continued hypertrophy of the cystoma around the ovary and atrophy in the prostate gland. In addition, it was revealed that chemical substances within WSF have estrogenic activity. A major component of heavy oil, polycyclic aromatic hydrocarbons (PAHs), is known to present estrogenic activity. It is likely that the cystoma-like formation in female mice and atrophy of prostate gland in male resulted of estrogenic substances present in the WSF which might be the PAHs.

Key words: Heavy oil, Oral administration, Cystoma-like formation, Prostate gland atrophy, Estrogenic activity

INTRODUCTION

The contamination of heavy oil through oil tanker spillage or more catastrophic accidents constitutes a continual and serious threat to the marine environment. In recent years, a wreck in the Sea of Japan involving the Russian tanker, Nakhodka, released a large quantity (> 6,000 kl) of heavy oil which spread along the coastline of the northern part of Japan (Kizu et al., 1998). Heavy oil spill severely affects the ecosystem around the accidental site. The released oil emulsion in small droplets in the column of seawater may drift across middle phase or reach the benthos. In middle or sediment phase-dwelling fishes, oil droplets caused severe stress and damage (Alkindi et al., 1996; Song et al., 2008). Many studies examined the influence of this event on the marine ecosystem, including effects on mussels, fish and seabirds (Lemiere et al., 2004; Nakayama et al., 2008; Alonso-Alvarez et al., 2007). Some chemical components that a fraction of the heavy oil is soluble in water may affect the organisms (Navas et al., 2006). Though many reports described the influence of heavy oil on marine organisms, few studies have investigated its effects on the health of mammals and humans.

Some chemical compounds were found to be bioconcentrated in fish-eating seal through the marine food chain.
for a long time (Neale et al., 2002). Hence, although the risk of direct exposure to heavy oil may be low, there still remains a real risk of accumulation of some heavy oil substances or compounds within marine organisms, which would pose a potential threat via the food web. Especially, we can predict that Japanese have a high risk of chemicals accumulating in their body because of their large intake of many kinds of fishes.

Some heavy oils are characterized by a high content of polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene present in heavy oil (Hayakawa et al., 2006; Sacco-Alvarez et al., 2008). PAHs rank as relatively soluble and more soluble than alkanes having an equal number of carbon atoms (McAuliffe, 1987; Ramachandran et al., 2006). These chemicals act as carcinogens or mutagens (Arcaro et al., 2001). Therefore, exposure to heavy oil components is potentially hazardous to our health. However, there is little research on the hazardous effects of heavy oil components on human health when these substances are orally taken through polluted food.

In this research, we investigated the potential influence of the chemical substances occurring in heavy oil in vivo, using mice as a mammalian model animal. We focused on the toxicity of water-soluble substances found in heavy oil. Water-soluble fraction (WSF) was orally administrated to mice, and the toxicological effects on mammals were assessed. In addition, to hypothesize the contributing chemical substances, we examined the relationship between the estrogenic activity in WSF and pathological effects.

MATERIALS AND METHODS

Sample
Water-soluble fraction was extracted from heavy oil with water by the method described previously (Stephens et al., 1997). Briefly, heavy oil was suspended in distilled water at 10% (v/v) and gently mixed for 20 hr. Following centrifugation, the water phase was collected, hereafter termed as water-soluble fraction, WSF. The sample solution was kept at -20°C and thawed at room temperature prior to administration. This sample was used to supply an in vitro assay system and administration experiment.

Animals and oral administration
Albino mice were purchased from the Japan SLC, Inc. (Shizuoka, Japan). Mice were housed in a room maintained at 24°C on a 12-hr light/dark cycle in a specific pathogen-free facility, and provided tap water and diet ad libitum. All experiments using mice described herein were approved by the Ehime University Animal Care and Use Committee and were performed in accordance with applicable guidelines and regulations.

For oral administration, three mice per group were administrated WSF or distilled water as a control. The first administration of WSF was performed in 8-week-old female and 12-week-old male mice, because they were sexually mature. In order to investigate the pathological effects of the WSF, all mice were administrated 20 μl volume (1 ml/kg) of WSF at various concentrations everyday for 28 days (Breinholt et al., 2000). After measurement of body weight, mice were killed by cervical dislocation, and liver and kidneys excised at Day 7, 14, 21 and 28.

To examine pathological effects on reproductive organs, ovaries and prostate glands were also excised. After washing with phosphate buffered saline (PBS) twice, the total weight of each tissue, with the exception of ovaries, was measured. The mean tissue weight of the three control mice was calibrated as 100%. Relative atrophy rate of each tissue was then calculated from the experimental mouse values against the control. This administration experiment was independently performed twice.

Reporter gene assay
To examine whether or not the chemical substances in WSF have estrogenic activity, luciferase reporter gene assay was performed using T47D-KBluc cells purchased from ATCC (Manassas, VA, USA). T47D-KBluc cells are sensitive and responsive to estrogen cell line, stable transfected with estrogen-responsive luciferase reporter construct (Wilson et al., 2004). These cells were normally maintained in ERDF medium (Kyoluto pharmaceutical, Tokyo, Japan) supplemented with 5% Fetal bovine serum (FBS) at 37°C under humidified 5% CO₂-air. FBS was treated with 50 mg/ml activated charcoal (Sigma, St Louis, MO, USA) for 0.5 hr. Charcoal-treated FBS (cFBS) was filtrated by a 0.22 μm membrane filter. The cFBS was then used for estrogen assay by using estrogen-responsive T47D-KBluc cells.

Three days prior to the estrogen assay, FBS in the culture medium was replaced by cFBS. T47D-KBluc cells were pre-cultured ERDF medium supplemented with 5% cFBS according to the condition described above. Following pre-culture, cells were inoculated at 2x10⁶ cells/ml in 5% cFBS-ERDF medium and treated with WSF at various concentrations for 48 hr. Culture treated with 17ß-estradiol (Sigma) at 0.1 nM was used as control. After 48 hr, luciferase activity was determined in the cells using a commercial kit (Promega, Madison, WI, USA). Luminiscence was measured by Luminescencer-JNR AB1200 (ATTO, Tokyo, Japan) according to the instructions provided by the manufacturer.
Statistical analysis

Significance of differences between means was assessed using Tukey's test. Each value of $p < 0.05^*$ or $p < 0.01^{**}$ was considered statistically significant.

RESULTS AND DISCUSSION

Many previous studies have found various chemical constituents in heavy oil (Boffetta et al., 1997; Andersen et al., 2008; Guitart et al., 2008). However, the influences of WSF on mammals remain unclear. For the study, mice were used as model to determine the potential toxicity of the substances contained in WSF.

In order to investigate the pathological effects of the WSF, mice were orally administrated WSF every day for 28 days. At first, we focused on whether the absorption of WSF has an influence on body weight. WSF-administered 8-week-old female and 12-week-old male mice showed normal behavior and no mortality within the administration periods. As shown in Fig. 1A, the body weight of WSF-administered female mice was slightly reduced along the course of the administration period, whereas male mice showed no changes throughout the treatment course (Fig. 1B), indicating a greater effect WSF on female mice. Presently, we cannot clearly explain why only the body weight of WSF-administered female mice for 21 days indicated significant difference with control. However, body weights of WSF-administered mice in other periods showed obvious weight-loss tendency compared to control group in two independently conducted experiments. Female mice may therefore be more sensitive to chemical components present in WSF.

To examine pathological effects on mice, WSF was orally administrated to mice for 28 days. It was thought that the influence of the WSF may be manifested as atrophy of liver and kidney tissues, the two major organs responsible for detoxification. Our results showed weight loss in liver tissues of WSF-administered mice, however, kidney weights were not affected by the WSF (Table 1). The results in all mice showed a similar tendency among tissues.

Furthermore, we found abnormalities in genital organs. An appreciable fluid accumulation around the ovary was observed in the WSF-administered mice, consistent with symptoms similar to a condition termed cystoma (Part, 1999; Dietel and Hauptmann, 2000). At day-7, cystoma-like formation occurred in approximately 80% of WSF-administered female mice, and appeared in all WSF-administered female mice by at least 14 days after administration (Fig. 2A). As indicated in Figs. 2A and B, hypertrophy of cystoma was observed in some of WSF-administered mice from two independently conducted experiments. Some clinical reports have revealed that hypertrophic cystoma progresses to ovarian tumors (Fox, 1993; Eroschenko et al., 1995; Part, 1999). However, ovarian tumors were not found in WSF-administered female mice during the 28-day period. In addition, the dose-dependent effect of WSF on female mice was examined (data not shown). The results indicated that oral administration of WSF at lower concentrations (1/100) also caused cystoma-like formation in female mice.

The effects of WSF on genital organs of male mice indicated a shrinking of the prostate gland (Figs. 2C and D). Atrophy of the prostate gland proceeded according to administration period with the weight of the prostate administrated mice from two independently conducted experiments.

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Table 1. Various tissue weight in WSF-administrated male mice

<table>
<thead>
<tr>
<th>Administration period</th>
<th>Liver</th>
<th>Kidney</th>
<th>Prostate</th>
<th>Testis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>WSF</td>
<td>Control</td>
<td>WSF</td>
</tr>
<tr>
<td>7 days</td>
<td>2.49 ± 0.15</td>
<td>1.94 ± 0.09 **</td>
<td>0.85 ± 0.01</td>
<td>0.87 ± 0.04</td>
</tr>
<tr>
<td>14 days</td>
<td>2.22 ± 0.07</td>
<td>1.88 ± 0.03 **</td>
<td>0.77 ± 0.03</td>
<td>0.76 ± 0.03</td>
</tr>
<tr>
<td>21 days</td>
<td>2.34 ± 0.06</td>
<td>2.10 ± 0.07 **</td>
<td>0.83 ± 0.04</td>
<td>0.83 ± 0.07</td>
</tr>
<tr>
<td>28 days</td>
<td>2.53 ± 0.12</td>
<td>2.14 ± 0.04 **</td>
<td>0.82 ± 0.02</td>
<td>0.80 ± 0.01</td>
</tr>
</tbody>
</table>

(unit of weight: g, n = 3, **: p < 0.01, *: p < 0.05)

Fig. 2. Cystoma-like formation around the ovary was caused by WSF-administration in female mice (A and B). Atrophic prostate gland occurred by WSF-administration in male mice (C and D). Upper and lower panels show female and male mice, respectively. Right and left side in each panel are tissues of WSF-administrated and control mice, respectively. Arrowheads indicated abnormal region. Administration experiment was independently carried out twice.

With regards to cystoma-like formation in female, and prostate gland atrophy in male mice, it is thought that chemical substances in WSF may have estrogenic activity (McClain et al., 2005). It is known that estrogen can shrink the prostate gland, and is used for treatment of prostatism (Geller, 1992; Roberts, 1966). To examine whether or not WSF have estrogenic activity, luciferase reporter gene assay was carried out using estrogen-responsive T47D-KBluc cells. As shown in Fig. 3, WSF induced the expression of the luciferase gene in T47D-
WSF induced cystoma-like formation and prostate gland atrophy

Fig. 3. WSF has estrogenic activity shown by luciferase assay. Luciferase reporter gene in T47D-KBluc cells was expressed at each WSF concentration (•) compared with 0.1 nM 17β-estradiol (○) as a control. The highest estrogenic activity was approximately 1.8-fold at 0.15% of WSF concentration. Experiments were repeated 3 times under various WSF concentrations.

KBluc cells. Luciferase levels in T47D-KBluc cells treated with WSF were higher than those treated with 17β-estradiol (0.1 nM). This result clearly indicates that WSF contains substances having estrogenic-like activity.

As indicated herein, chemical substances in heavy oil eluted with distilled water caused various physiological disorder in mice such as atrophy of the prostate gland or cystoma-like formation in ovaries. Although it is not known why atrophy or cystoma-like formations occurred in genital tissues, it is possible that estrogenic active substances of the WSF induced abnormalities in reproductive tissues of both male and female mice (Singhal et al., 2008). It is generally known that PAHs exhibit characteristics such as having complex chemical structure and water-soluble. However its concentration in water column would increase in low salinity coastal water or estuaries (Ramachandran et al., 2006). It follows that some PAHs may be included in WSF prepared from heavy oil (Hayakawa et al., 2006; Saco-Alvarez et al., 2008).

Furthermore, most chemicals among PAHs such as benzo[a]pyrene or benzo[a]anthracene or phenolic compounds have shown estrogenic activity (Charles et al., 2000). Especially, some PAHs have carcinogenicity (Singhal et al., 2008), and these compounds may be one of the candidates that caused cystoma-like formation around the ovary. PAHs have been observed to activate estrogen receptor (ER) alpha in a reporter gene assay inducing ER-dependent cell proliferation in breast cancer cells (Plíšková et al., 2005; Vondráček et al., 2002). In ovaries of mice exposed to benzo[a]pyrene, the level of marker protein, which increased upon induction of genotoxic damage and apoptosis, was up-regulated. (Kwon et al., 2002; Kummer et al., 2008). It suggests that the water-soluble chemicals may alter the endocrine system by its estrogenic properties. Indeed, two kinds of ERs were expressed in the normal prostatic epithelial cells (Lau et al., 2000), which have an important function in genital cell development. ER alpha and ER beta-disrupted females have been shown to be sterile (Dupont et al., 2000). Therefore results from this study suggest that PAHs such as benzo[a]pyrene in WSF may lead to abnormalities in genital organs or signaling transduction through the ERs. However, further study is needed to reveal what kinds of chemicals in WSF induce abnormalities of these tissues.

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