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Risk Assessment of Heavy Oil on Terrestrial Mammals

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Abstract—In the advent of accidental oil spills, such as tanker wrecks, a large quantity of heavy oil is released into the marine environment. The environmental pollution by heavy oil is regarded as serious problems, and has various influences on the marine ecosystem. The influences of the chemical substances in heavy oil are widely studied on many kinds of marine organisms. On the other hand, the effects of chemicals in heavy oil on human health have not yet been cleared. Therefore, we investigated the pathological effects of the substances in heavy oil extracted in distilled water on mice. Substances in heavy oil were extracted in distilled water, designated as water-soluble fraction (WSF). WSF was orally administrated to female mice for seven days. Interestingly, cystoma-like formation was observed on around the ovary in the mice administrated WSF. Additionally, it was revealed that WSF has estrogen-like activity. From these results, it is suggested that estrogen-like substances in WSF induced the development of ovarian cystoma in mice.

Keywords: heavy oil, water-soluble fraction (WSF), oral administration, cystoma-like formation, atrophy

INTRODUCTION

The environmental pollution by heavy oil causes serious influence on the marine ecosystem. As one of the causes, a high incidence of heavy oil spills by shipwrecks of heavy oil tankers are mentioned. In recent years, a Russian tanker, the Nakhodka was wrecked in the Japan Sea. Over 6000 kL of heavy oil leaked from this accident was spread along with the coastline of northern part of Japan. The influence of this catastrophic event on marline ecosystem was investigated by many researchers (Kizu *et al*., 1998; Hayakawa *et al*., 2006). The influence was examined at various viewpoints on marine ecosystem, typically the targets about mussel, fish and sea birds. On the other hand, there are few reports on the influence of the heavy oil for our health. Although direct exposure of heavy oil itself may be low, it can not be ignored that there is a possibility that the high

concentration of chemical substances contained in heavy oil can occur through bioaccumulation. In this research, we investigated the potential influence of the chemical substances contained in heavy oil *in vivo*, using mice as a terrestrial mammalian model.

Polycyclic aromatic hydrocarbons such as benzopyrene are well known as chemicals contained in heavy oil (Hayakawa *et al*., 2006; Saco-Alvarez *et al*., 2008). These chemicals act as carcinogens or mutagens (Arcaro *et al*., 2001). Therefore exposure to heavy oil can be a serious health hazard. We focused on the toxicity of water-soluble substances in heavy oil, also including the second metabolite change on terrestrial mammals. Water-soluble fraction (WSF) was orally administrated to mice, and the toxicity of the fraction was assessed.

MATERIALS AND METHODS

Sample

Heavy oil was kindly distributed by Dr. Kitamura. The heavy oil was extracted with distilled water. Briefly, heavy oil was added to distilled water (10%, v/v), gently mixed for 20 hrs using rotator (Stephens *et al*., 1997). Collected water phase was designated as water-soluble fraction (WSF), retained for the study.

Animal and oral administration

Female albino mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Mice were housed in a specific pathogen-free (SPF) facility at Integrated Center for Science, INCS, Ehime University. For administration assay, female mice for 6 weeks age were used. WSF were administrated to 20 μl per mouse per day for 7 days (Breinholt *et al*., 2000). All mice were killed by cervical dislocation; ovary and a part of uterus were excised for pathological effect comparison. All animal experiments were carried out in accordance with protocols approved by the Ehime University Animal Care and Use Committee.

Cells and culture condition

T47D-KBluc cells were purchased from ATCC. T47D human breast cancer cells, which constantly express estrogen receptor (ER) alpha and beta, were stably transfected with estrogen-responsive elements (ERE)-promoter-luciferase reporter gene construct (Willson *et al*., 2004). T47D-KBluc cells are sensitive to 17β-estradiol (E2), and were subcultured in ERDF medium with 10% fetal bovine serum (FBS) at 37°C under 5% CO_2 in humidified air condition.

Luciferase assay

T47D-KBluc cells were employed to investigate estrogen activity of heavy oil extract by luciferase assay. Briefly, after the cells pre-cultured with charcoal-treated FBS for 3 days, cells were cultured in 5% charcoal-treated FBS/ERDF

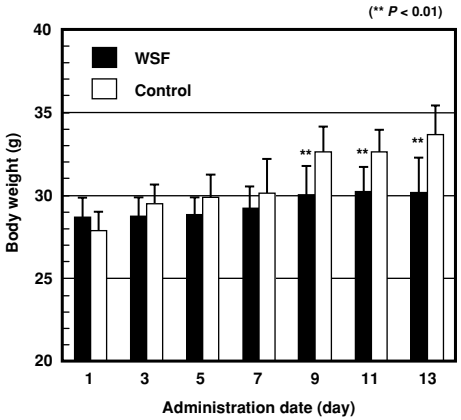

Fig. 1. Measurement of body and tissue weights on WSF-administrated mice. Body weight of mice were measured at constant time of 1, 3, 5, 7, 9, 11 and 13 days. Results were twice independent experiments (n = 3). Asterisks indicate the significant differences (**P < 0.01).

medium supplemented with WSF for 48 hrs. The luciferase assay was carried out using the Luciferase Assay System (Promega, USA) and Luminescencer-JNR (ATTO, Japan) according to the instructions provided by manufacturer.

RESULTS AND DISCUSSION

Effect of WSF on mouse body weight

In order to investigate the pathological effects of heavy oil extracts, a mouse was administrated 20 μl of WSF per day for 14 days (Breinholt *et al*., 2000). At first, we focused whether WSF had the influence on body weight gain with the growth. Body weights of WSF-administrated mice were measured at 1, 3, 5, 7, 9, 11 and 13 day. WSF-administrated mice did not cause abnormal action or sudden death. The quantity of feeding and drinking water did not changed. As shown in Fig. 1, the body weight of WSF-administrated mice was slightly decreased along with administration period. After Day 9, the body weight of WSF group was significantly lower than that of control group. This result showed that the chemical substances contained in WSF were induced body weight loss with growth.

Pathological effect of WSF

To examine the pathological effects on mice, WSF was orally administrated to mice for 14 days. All mice were physically euthanized by cervical dislocation for the examination of internal organs. Liver, kidney, spleen and thymus were removed from abdominal cavity. After a wash with PBS, each tissue was blot-dried on surface and whole weight was measured. Liver and kidney are antidotal

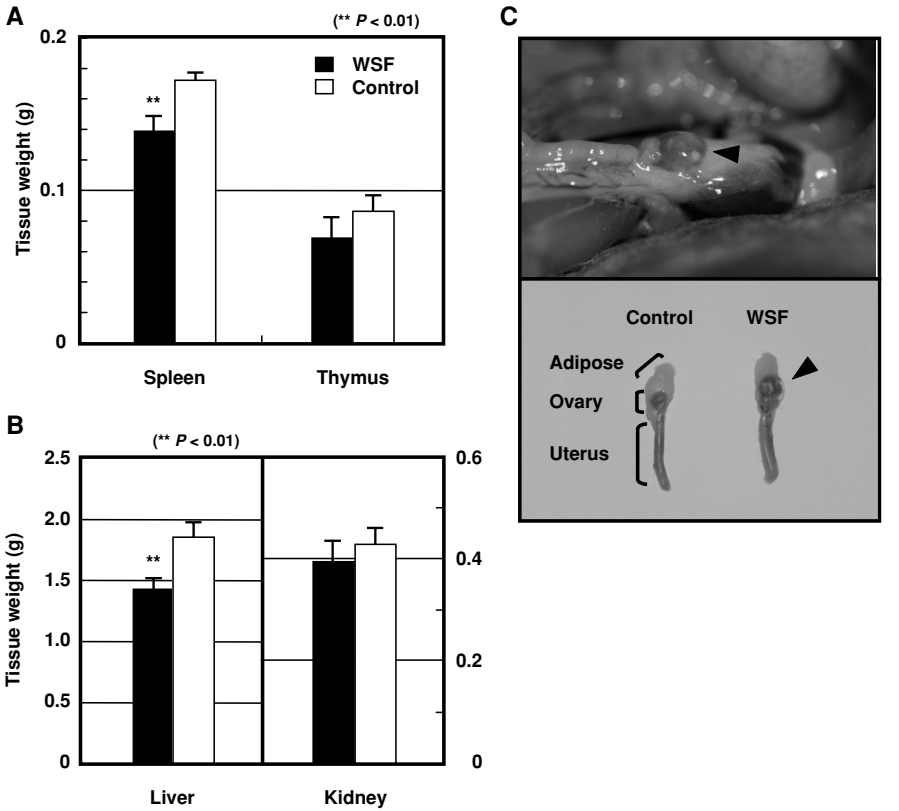

Fig. 2. Pathological effects on ovary in WSF-administrated mice. At day 14, WSF or distilled water as control-administrated mice were sacrificed. Spleen, thymus, liver and kidney were excised from abdominal cavity, and trimmed away the connective tissue. Tissue weight of spleen and thymus or liver and kidney are shown in A and B, respectively. C, Cystoma-like formation around ovary was found in WSF-administrated mice for 14 days (upper panel); ovary and a part of uterus are shown in the lower panel. Arrows indicate ovarian cysts. Results were twice independent experiments ($n = 3$). Asterisks indicate significant differences (**$P < 0.01$).

and metabolizing tissue against toxic or chemical substances taken in body. It was supposed that influence of chemicals might be observed as the atrophy in liver and kidney responsible for detoxification of chemicals. As shown in Fig. 2A, tissue weight of liver was significantly lower than that of control, approximately 77% vs. control. However, kidney weight was same level between WSF group and control. In addition, immune tissues were compared. As indicated in Fig. 2B, spleen and thymus shrunk and were induced remarkable weight loss. Atrophy in immune tissue might be resulted in serious immune deficiency.

Moreover, we searched for other tissues exhibiting abnormality. Surprisingly, serous fluid accumulation around the ovary was observed in WSF-administrated

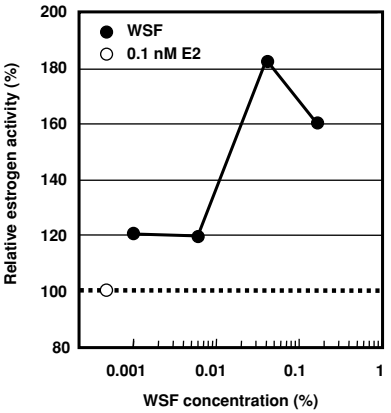

Fig. 3. Estrogen-like activity in WSF. T47D-WSF concentrations for 48 hr. Luciferase reporter gene was expressed at each concentration compared with 0.1 nM β-estradiol as a control. The highest estrogen-like activity was indicated approximately 1.8-fold at 0.04% of WSF concentration.

mice. This abnormality was similar to cystoma disease, termed cystoma-like formation. As shown in Fig. 2C, hypertrophy of cystoma was observed in some of WSF administrated mice. Cystoma-like formation was caused in approximately 80% of WSF-administrated mice. In addition, hemorrhage around the ovary was found in WSF-administrated mice. The dose-dependent effect of WSF on mice was examined. WSF was diluted with distilled water at 10^n (n = 0–2). WSF exposure at lower concentration of WSF (10^2 dilution) by oral administration also caused cystoma-like formation in mice (data not shown).

From these results, it was suggested that the chemical substances contained in WSF attack the several tissues and induce pathological disorder such as inflammation and hemorrhage. Recently, clinical reports have revealed that hypertrophic cystoma can result in ovarian tumor (Eroschenko *et al*., 1995). However, ovarian tumors were not found in WSF-administrated mice during the 14 days of this study. At present, we do not understand the physiological response of why atrophy of tissues occurred in liver and immune tissues. To examine this a detailed analysis of this mechanism by cell population analysis is warranted.

Chemical substances contained in WSF have estrogen-like activity

Concerning cystoma-like formation in mice, it is supposed that chemicals in WSF have estrogen-like activity. To examine whether chemical substances in WSF have the estrogen-like activity, luciferase reporter gene assay was carried out using estrogen-responsive T47D-LBluc cells. T47D-LBluc cells were pre-cultured in ERDF medium supplemented with 10% charcoal-treated FBS for 3 days. After pre-culture, T47D-KBluc cells were cultured at 2×10^5 cells/ml in culture medium containing WSF at several concentrations. Following 48 h cultivation, the expression level of luciferase was measured by luciferase assay.

As shown in Fig. 3, WSF induced the expression of luciferase gene in T47D-KBluc cells. Relative luciferase unit (RLU) level in T47D-KBluc cells treated with WSF was higher than that of 0.1 nM E2 as positive control at all concentrations of WSF. This result suggests that WSF contains substances possessing estrogen-like activity.

From these results, chemicals extracted with distilled water from heavy oil were caused several influences in mouse body: atrophy of liver, spleen and thymus, and cystoma-like formation in ovary. On the other hand, WSF contains estrogen-like active substances. It is assumed that the target of chemicals contained in WSF may be the reproductive or immune cells expressing estrogen receptor. Further study will try to investigate what specific chemicals in WSF induce abnormality of these tissues.

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