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Letter

Heavy oil fraction induces the dysplastic sperm in male mouse

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ABSTRACT — Heavy oil is one of the most serious pollutants in marine ecosystem. The poisonous influences of the chemical substances contained in heavy oil on many kinds of marine organisms are widely studied. However, the influence of the chemical compounds in heavy oil on our health has not been cleared yet. In order to reveal the poisonous influences of these chemical compounds on mammalian reproductive system, water-soluble fraction (WSF) extracted from heavy oil was administrated to mice for 2 weeks. WSF-administrated mice were crossed with either WSF- or distilled water-administrated group for mating experiment. When WSF-administrated male mice were used as a father, it reduced not only mating ratio, but also neonatal male ratio. The numbers of sperms of WSF-administrated male mice were decreased. In addition, abnormality of sperms such as bent or twisted tail was increased approximately 6-fold by WSF intake. The level of testosterone in serum from WSF-administrated mice was lower than that from control mice. Testosterone is the most important for the spermatogenesis in vertebrate. It is supposed from these findings, the decrease in the number of sperms may relate with the reduction of sex hormone level in serum. It is suggested from these results that the chemical substances in WSF affected the sperm function in reproductive system of male mice.

Key words: Water-soluble fraction (WSF), Oral administration, Breeding, Spermatogenesis, Testosterone

INTRODUCTION

The environmental pollution by heavy oil is a serious problem for marine ecosystem, and compounds in heavy oil have various harmful influences on many kinds of marine organisms (Lemiere *et al.*, 2004; Nakayama *et al.*, 2008). On the other hand, the effects of chemicals contained in heavy oil on our health, especially on the reproductive system have not yet been clarified. Though the risk of the direct exposure to the chemicals in heavy oil may be low for human, it cannot be ignored that there is a possibility that we may take the highly concentrated chemicals via food web.

We previously found that the chemical compounds contained in water-soluble fraction (WSF) induced the atrophy of spleen and thymus in mice (Nishimoto *et al.*, 2008). In addition, the activity of immunoglobulin production in lymphocytes were down-regulated by WSF of heavy oil (Nishimoto *et al.*, 2009a). Furthermore, the chemical compounds in WSF developed not only cystoma in female, but also shrinkage of prostate gland in male (Nishimoto *et al.*, 2009b). In this study, we focused on the toxic effects of chemical compounds contained in heavy oil on the mouse reproductive system.

The abnormality of sperm is caused by various factors. Some of the environmental chemical substances such as

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$NaNO_3$ or Perfluorinated alkyl acids induced the testicular toxicity in rat (Aly *et al.*, 2010; Chemes and Rawe, 2010; Feng *et al.*, 2010). Moreover, ethanol induced the depression of fertility and impairment of sperm (Anderson *et al.*, 1983).

Spermatogenesis is a very significant process for the alternation of generation. In this process, testosterone plays an important role in the formation and maturation of sperms. Gonadotrophin (luteinizing hormone (LH)) secreted from pituitary gland regulates the Leydig cells. Leydig cells, the testosterone producing cells in the mammalian testis, are differentiated cells that rarely proliferate in the adult (Saez 1994; Chen *et al.*, 2010). Leydig cells undergo a process of morphologic and functional transformations (Haider, 2004). The stimulation by LH promotes the testosterone of synthesis and production in Leydig cells in niche among the seminiferous tubules. Secreted testosterone is supplied to sertori cells, and these cells induce development of spermatoblasts (Ruwanpura *et al.*, 2010).

In this article, we report that the chemical compounds in WSF give rise to the depression of sexual activity, bent sperm tail, and reduction of testosterone in serum.

MATERIALS AND MERHODS

Sample preparation

Heavy oil (bunker C) obtained from an oil company was used for exposure experiments. Heavy oil was extracted with distilled water (DW). Three ml of heavy oil was suspended in 30 ml of DW, and gently mixed for 20 hr following the methods described previously (Stephens *et al.*, 1997). Collected water phase sample was called "10% (v/v) water-soluble fraction (WSF)". WSF was used for oral administration to mice.

Animals

ICR 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 specific pathogen-free facility, and provided tap water and diet *ad libitum*. All animal experiments were carried out in accordance with protocols approved by the Ehime University Animal Care and Use Committee and were performed in accordance with applicable guidelines and regulations.

Oral administration and Breeding experiment

Three mice (8-week-old) per group were administrated WSF or DW as a vehicle control. Each mouse was administrated with 0.667 ml of WSF per kg per day for 14 days continuously. For breeding experiment, as shown in Fig.1, all mice were divided in 8 groups. To prevent fighting, male mouse was individually kept in cage. Following WSF or DW administration, female mouse was mated with male mouse in the afternoon on day 14. The next morning, female mouse was separated from male mouse, and the number of plug-positive female mice of each group was counted. After 20 days from mating, all neonates were born by natural parturition. The number of newborn mice of each group was counted at the ablactation point of 28 days.

Sperm observation

Five male mice per group were administrated WSF or DW for 28 days. All mice sacrificed by cervical dislocation, and epididymides were enucleated from the scrotums. Mature sperms were collected from epididymal follicles. Epididymides were tore in 1 mM EDTA-PBS, and packed sperms were incubated to recover migration for 30 min. Micrographs of sperms were captured by CCD-camera attached to microscope (Shimadzu Rika, Tokyo, Japan) according to the manufacturer's instruction.

Quantification of testosterone

Male mice (n = 5) per group were administrated WSF or DW for 28 days. Whole blood was collected from each mouse, and serum was separated from whole blood. The amount of testosterone in serum was measured by using Testosterone ELISA TEST (Endocrine Technologies, Inc., Newark, CA, USA), according to the instructions provided by manufacturer.

Statistical analysis

Results are expressed as means ± standard deviation (S.D.). Tukey's test was used to assess the statistical significance of the difference between control and WSF-administrated group.

RESULTS AND DISCUSSION

In order to observe the effect of WSF on mouse sexuality and genital potential, mating examination was performed using WSF-administrated mice. Mice were administrated WSF or DW for 2 weeks, and crossed with either WSF- or DW-administrated mice. The mating pattern was indicated in Fig.1. Cystoma was observed in approximately 80% of WSF-administrated female mice and atrophy of prostate gland was in the same of WSF-administrated male mice (Nishimoto *et al.*, 2009b). The mating ratio was obviously decreased when WSF was administrated to male mice (Table 1). Interestingly, the

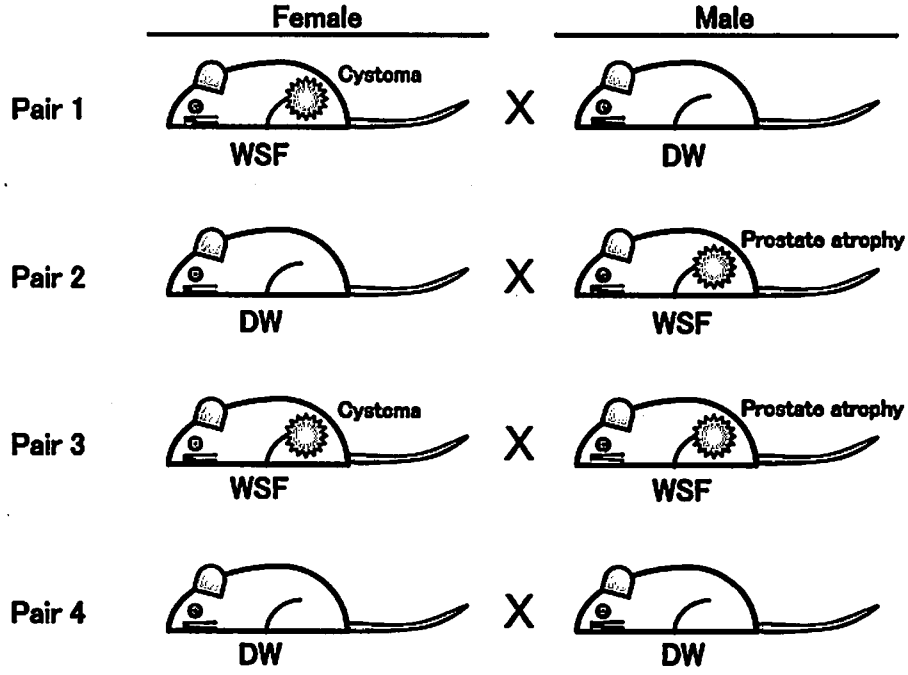

Fig. 1. Mating experiment of WSF-administrated mice. Scheme of cross pattern for mating experiment. WSF- and DW (vehicle control)-administrated mice were crossed with each other. The cystoma was observed in WSF-administrated female mice, and the atrophy of prostate gland was observed in WSF-administrated male mice.

Table 1. Results of mating experiment

Female × Male	Mating ratio (%)	Neonatal male/female (%)
WSF × DW	75.0	140.0
DW × WSF	37.5	75.0
WSF × WSF	25.0 *	60.0
DW × DW	75.0	135.2

The mating ratio was calculated by the number of plug-positive female mice against total female mice. Neonatal sex ratio was calculated by the number of male against female at 4 weeks old. Asterisk indicates that the data are significantly doffernt from the DW × DW data. $^*p < 0.05$.

sex ratio between male and female in newborn mice was 3 : 5, when WSF-administrated male mice were used as a father. This result suggests that oral administration of WSF affects the reproductive system in male. Therefore, it is supposed that chemical compounds in WSF may be poisonous toward sperms in male.

To investigate the effects of WSF on reproductive system of male mice, male mice were orally administrated WSF at 0.667 ml/kg/day for 28 days. Both body and testis weight of mice was not affected by WSF (data not shown). The epididymides were extracted from the lower abdomen, and were torn in 1 mM EDTA-PBS. In order to restore migratory potential, packed sperms were incubated at 37°C for 30 min. As shown in Fig. 2, the number of mature sperms from WSF-administrated mice was remarkably decreased in comparison with control mice. Interestingly, the tail of many sperms bent or twisted in the middle, and wound into an L-shape (Fig. 3A). Moreover, the rate of abnormal sperms increased approximately 8-fold by intake of WSF (Fig. 3B).

In general, it is well-known that vitality of Y chromosome sperm is weak in comparison with one of X chromosome sperm. From these results, it was suggested that experimental data indicated in Table 1 was caused by not only the decreased number of all sperms, but also the increased number of the deformed sperms.

Testosterone is one of the male sex hormone, and it is produced by Leydig cells located in the niche of seminiferous tubules. Testosterone is mainly functioned from ini-

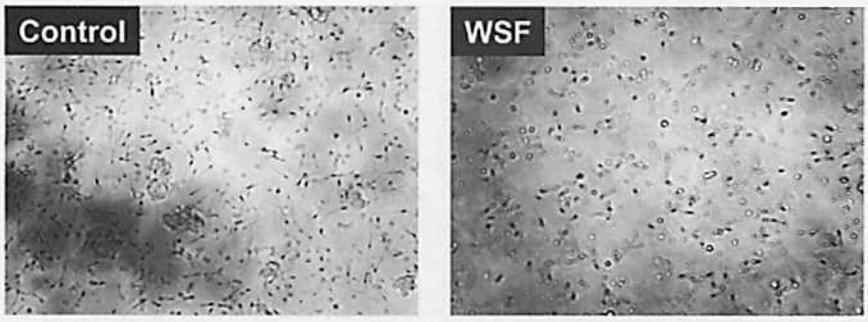

Fig. 2. Observation of mature sperms in WSF-administrated male mice. Mice were administrated with WSF or DW for 28 days. Mature sperms were collected from ruptured epididymis. To recover the migration potential, sperms in 1mM EDTA/PBS were performed incubation for 30 min. The images were arbitrarily taken.

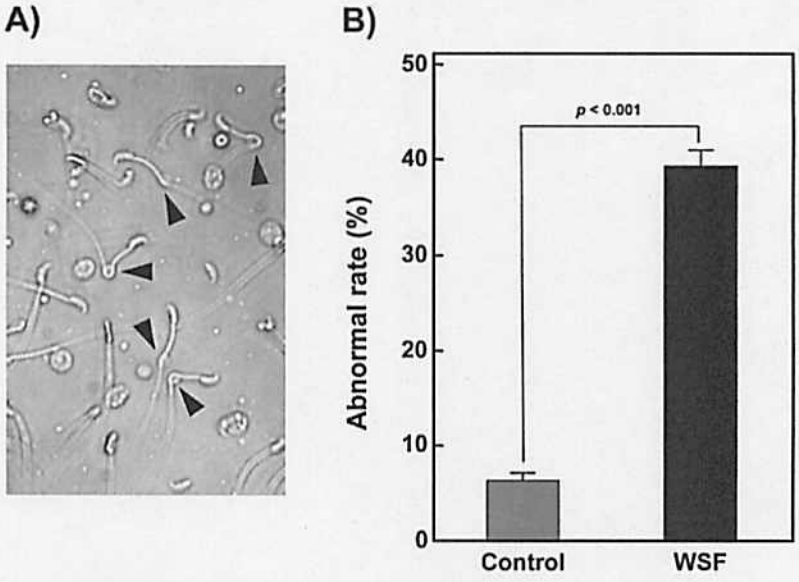

Fig. 3. Abnormal sperms in WSF-administrated male mice. Mice were administrated with WSF or DW for 28 days. A, Abnormal sperms. Arrowheads indicated the bent points. B, Abnormal rate was calculated by anomaly sperms against total sperms in captured images. These results are expressed as the mean ± S.D. of five independent measurements.

tiation until maturation through the spermatogenesis (Lui and Lee, 2009). To observe the effect of WSF on Leydig cells, testosterone in serum was measured by ELISA. The testosterone level of WSF-administrated mice was markedly reduced against control mice (Fig. 4). It may suggest from the result that the water soluble chemical compounds in heavy oil attacked the Leydig cells in testis as one of the target. It is worth while examining the subject more closely.

There is the blood-testis barrier (BTB) system in male, protecting the testis from detrimental drugs and xenobiotics against spermatogenesis (Murk *et al.*, 2011; Su *et al.*, 2011). From the results indicated here, it is supposed that WSF uptake suppresses the testosterone synthesis or production in Leydig cells. The chemical compounds contained in WSF may break the BTB system and slip through it. In conclusion, our findings suggest that the water soluble chemical compounds in heavy oil induce the disruption in male reproductive system.

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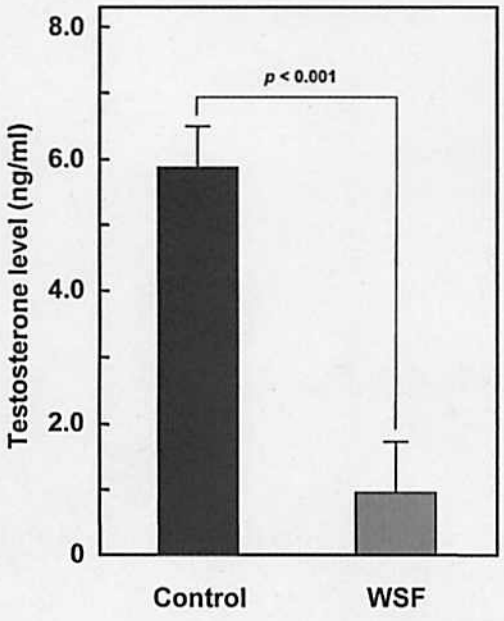

Fig. 4. Quantification of testosterone in serum from WSF-administrated male mice. Mice were orally administrated with WSF or DW for 28 days. Sera were prepared from whole blood. The sample solution, which diluted sera twenty times, was measured by ELISA. This experiment was carried out twice independently. These results are expressed as the mean ± S.D. of three independent measurements.

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