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Naphthalene-Induced Cytotoxicity on the Hepatopancreatic Cells of the Red Swamp Crayfish, *Procambarus clarkii*

R. Sarojini, P. S. Reddy, R. Nagabhushanam, and M. Fingerman

Department of Ecology, Evolution, and Organismal Biology, Tulane University, New Orleans, Louisiana 70118, USA

The regenerative capacity of the liver in mammals and the ability of mammalian hepatocytes to proliferate in culture are well documented (Leffert et al. 1979; Isom et al. 1985). After partial hepatectomy there is a prereplicative lag period before mitosis begins. Regeneration will continue until the original complement of liver tissue has been restored. The crustacean hepatopancreas, often also called the midgut gland, produces digestive enzymes, absorbs nutrients, and stores lipids. In some species the hepatopancreas also stores glycogen. However, the crustacean hepatopancreas does not perform all of the functions of the mammalian liver (van Weel 1974; Johnson 1980). The crayfish hepatopancreas, particularly of Procambarus clarkii, the species used in this investigation, has been extensively studied (Ogura 1959; Yamamoto 1960; Fingerman et al. 1965; Miyawaki et al. 1984). The hepatopancreas of Procambarus clarkii is a paired structure. Each half is divided into several lobes that consist of many blind tubules. These tubules empty into collecting ducts which eventually form the hepatopancreatic ducts that open into the gut. The tubules consist of four main cell types. The cells at the blind end of each tubule are called E-cells (Embryonalzellen). They are undifferentiated cells that differentiate into the other three cell types, which ultimately disintegrate after they have served their function. This ability of the hepatopancreas to replace lost cells is suggestive of the regenerative capability of the mammalian liver.

In this laboratory we have been studying the effects of naphthalene on molting of Procambarus clarkii (Sarojini et al. 1992). Naphthalene has been found to accumulate in the hepatopancreas in several crustaceans (Lee et al. 1976; Neff et al. 1976; Laurén and Rice 1985; Jaiswal and Sarojini 1990). The present study was undertaken to examine the effect of naphthalene exposure on the hepatopancreas of the red swamp crayfish, Procambarus clarkii, and given the presence of E-cells in the hepatopancreas, to determine in a quantitative fashion whether any damage to this organ by the naphthalene would be reversible during a period of depuration. A quantitative study of the cells in the hepatopancreas had not been done previously with any crustacean.

Send reprint requests to M. Fingerman at the above address.

MATERIALS AND METHODS

Three-hundred-twenty intermolt crayfish, Procambarus clarkii, each with a carapace length of 35 mm, were divided into 16 groups of 20 each. Eight groups were exposed to 10 ppm naphthalene. The naphthalene was first dissolved in acetone (30 mg/mL) and diluted with freshwater to provide the final desired naphthalene concentration of 10 ppm. Of the eight groups exposed to naphthalene, groups 1-5 were exposed for 1, 2, 3, 4 and 7 d, respectively; groups 6-8 for 15 d each. After 15 d naphthalene-exposed groups 7 and 8 were returned to clean freshwater for depuration. The remaining eight groups served as controls and were maintained in freshwater that contained the same concentration of acetone as in the naphthalene-containing solution. The crayfish were fed commercial crayfish food daily. The water in the control aquaria and the naphthalene-water in the experimental aquaria were changed daily.

At the end of each naphthalene exposure period (1, 2, 3, 4, 7 and 15 d for groups 1-6, respectively) and after 7 and 15 d of depuration for groups 7 and 8 that had been exposed to naphthalene for 15 d, the hepatopancreas was removed from each crayfish in the appropriate experimental group, weighed and processed for dissociation of the cells, cell counting and determination of dehydrogenase activity according to the method of Caldwell et al. (1993), with minor modifications. At the same time the hepatopancreas was removed from each crayfish in the corresponding control group. The concentration of the isolation medium was isosmotic with the blood of the crayfish (Huner and Barr 1991). Essentially, the method of Caldwell et al. (1993) consists of the following steps:

1. To dissociate the cells place an hepatopancreas in a 15 mL centrifuge tube containing 10 mL of the isolation medium (RPMI 1640 with sodium bicarbonate, EDTA (1.5 mM) and bovine serum albumin (1.0%)).
2. Seal the tube and incubate/agitate for 30 min at 37 °C.
3. Centrifuge for 5 minutes at 100 Xg.
4. Wash the pellet twice with 5 mL of incubating medium (RPMI 1640 with sodium carbonate, EDTA (1.5 mM) and bovine serum (0.1%) After the final wash, resuspend the cells in 1 mL of the incubating medium for use in Step 5.
5. Place 100 μL of the cell suspension and 100 μL of trypan blue in a polypropylene tube, and gently agitate to mix. Trypan blue is an exclusion dye used to distinguish viable cells from dead ones in suspension. Live cells do not take up this dye, whereas dead cells do.
6. Load a hemacytometer to capacity and count the live cells.
7. To assess cellular dehydrogenase activity after the cells are counted, 100 μL of equal concentrations of the cell suspensions of hepatopancreata of the naphthalene-exposed and control crayfish are transferred to wells in microtiter plate.
8. To this add 50 μL of MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide) prepared in a concentration of 2 mg/mL. The tetrazolium salt MTT interacts with mitochondrial components at several points along the respiratory chain (Slater et al. 1963). The result of this interaction is the

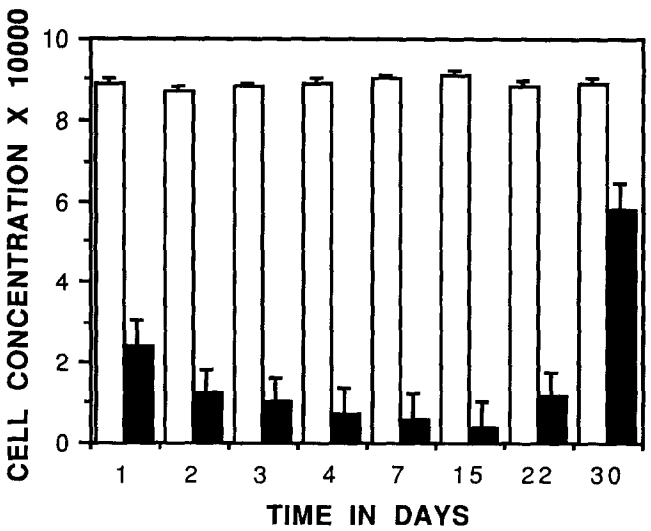

Figure 1. Mean concentration (cells per 100 μL of standard volume cell suspension) of live hepatopancreas cells of Procambarus clarkii. Solid bars, naphthalene-exposed; empty bars, control. Depuration began after Day 15. Error bars are SEM.

reduction of MTT by the succinate-tetrazolium reductase system to form a blue formazan dye which collects as crystals within the cell. This product can be dissolved into acid-isopropanol to give a colored solution which can be examined spectrophotometrically (Mosmann 1983). Conducting the assay in 96-well microtiter trays allows one to analyze the color formation on a spectrophotometer (ELISA reader) which can examine an entire 96-well tray in a 20-30-s time frame, therefore allowing a large number of samples to be tested within a day. The amount of formazan dye produced is proportional to cell number (Holt et al. 1987).

9. Incubate at 37°C with 5.1% CO_2 for 2 hr.
10. Carefully remove the supernatant without disturbing the cells.
11. Add 250 μL of 0.04 N HCl in isopropanol to each well.
12. Place each plate in a sonicator for 10 min.
13. Read O.D. at 490 nm on a microplate reader.

$$\text{Percent relative enzyme (dehydrogenase) activity} = \frac{\text{O.D. of sample}}{\text{O.D. of control}} \times 100$$

The data for each group of crayfish were averaged and used in the preparation of Figures 1 and 2.

RESULTS AND DISCUSSION

At the end of the first 24 hr of naphthalene exposure the mean live cell count of the exposed crayfish was 2.4 X 10^4 /100 μL versus 8.9 x 10^4 /100 μL for the controls (Fig. 1). The mean live cell count of the exposed crayfish continued to

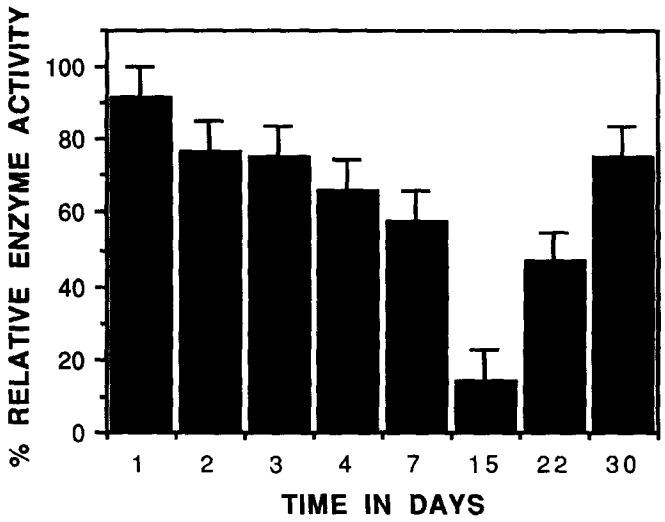

Figure 2. Mean percent relative enzyme (dehydrogenase) activity of hepatopancreas cells of crayfish exposed to naphthalene relative to the corresponding controls. Depuration began after Day 15. Error bars are SEM.

decrease throughout the 15-d exposure period, reaching a minimum of 3.8 x 10^4 /100 μL. After 7 d of depuration the cell concentration increased to 1.1 x 10^4 /100 μL, and after 15 d of depuration the count reached 5.8 X 10^4 /100 μL. Throughout the study the live cell concentration of the controls remained essentially constant. These results show that during the naphthalene exposure the number of live cells in the hepatopancreata of the exposed crayfish decreased markedly, and started to increase when the crayfish were returned to clean water.

Figure 2 shows the percent relative dehydrogenase activity of the cells in the hepatopancreata of the naphthalene-exposed crayfish in relation to the corresponding control crayfish. This enzyme activity is a measure of cell viability. The percent relative dehydrogenase activity after 24 hr exposure was 91.9%, and decreased to 76.9% and 75.2% after 48 hr and 72 hr exposure, respectively. By 15 d of exposure the percent relative dehydrogenase activity was only 14.2%. When the animals were depurated, the relative dehydrogenase activity increased to 46.5% after 7 d of depuration and to 75.0% after 15 d. During exposure to naphthalene the hepatopancreas underwent a significant weight loss (Fig. 3). After 24 hr exposure to naphthalene the average hepatopancreas weight decreased from 1113 mg to 810 mg. This weight loss continued throughout the 15 d exposure to naphthalene. After 15 d of exposure the weight averaged only 215 mg per hepatopancreas. When the animals were depurated, the average weight increased to 513 mg by 7 d, and 987 mg by 15 d.

In unpublished experiments in this laboratory, the hepatopancreas of Procambarus clarkii was found to accumulate a higher concentration of naphthalene during a

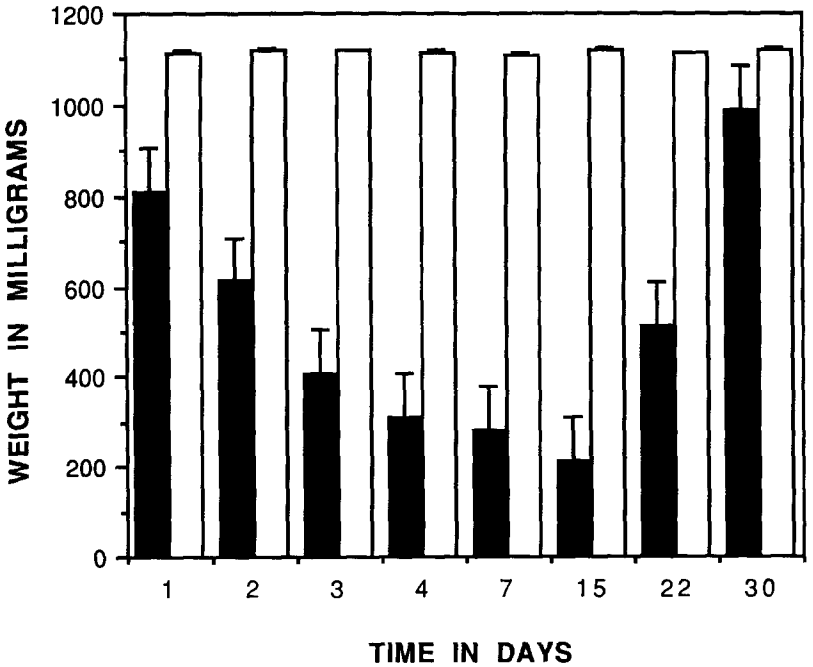

Figure 3. Mean weights in milligrams of the hepatopancreata of Procambarus clarkii. Solid bars, naphthalene-exposed; empty bars, control. Depuration began after Day 15. Error bars are SEM.

24-hr exposure period than any other organ in the crayfish. In the present study, we observed that the live cell concentration and percent relative dehydrogenase activity of the hepatopancreatic cells of the naphthalene-exposed crayfish progressively decreased during the exposure period (Figs. 1 and 2). After the animals were transferred to freshwater without naphthalene, the live cell counts of the hepatopancreatic cells of these crayfish that had been exposed to naphthalene and the percent relative enzyme activity of these cells progressively increased (Figs. 1 and 2). These results show clearly that naphthalene is toxic to hepatopancreatic cells of this crayfish. On the other hand, it is also clear that after exposure to naphthalene was discontinued, the hepatopancreatic cells were able to proliferate as evidenced by the increase in the live cell concentration during the depuration period (Fig. 1). In this respect, the hepatopancreas of the crayfish resembles the mammalian liver, both organs having the capability of replacing lost cells.

Jaiswal and Sarojini (1990) did not observe any cellular necrosis in the hepatopancreas of the freshwater prawn, Macrobrachium kistnensis, exposed to naphthalene. The hepatopancreas, however, did appear hyperactive after 24 or 48 hr of exposure to naphthalene as evidenced by the increase in the number and size of the secretory globules, but 72 and 96 hr exposures resulted in reduction of secretory activity in the tubules. Histological and histochemical studies of the

hepatopancreas of naphthalene-exposed Procambarus clarkii are now in progress in our laboratory and will at least give insight into the nature of the damage done to the tissue by this substance. This paper documents in quantitative terms for the first time the remarkable regenerative capability of the crustacean hepatopancreas.

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