

Material and Methods

Fish:

Tilapia (*Oreochromis niloticus*) with an average weight of 90 (60 – 120) g and average length of 20 (17 – 23) cm were obtained from a local hatchery (Fooky Fish Farm, Nowa, Shebin ElKanater, Qalyobya, Egypt). Fish were kept in tanks with aerated tap water at a temperature of 20°C. Fish were fed on commercial fish food, and acclimatized to laboratory conditions for a minimum of one week before the experiments were performed.

Rainbow trout (*Onchorynchus mykiss*) with an average weight of 90 (60 – 120) g and average length of 20 (18 – 22) cm were obtained from a local hatchery (Utah Division of Wildlife Resources, Springville Hatchery, Utah, USA). Fish were kept in tanks with aerated, charcoal-filtered circulating tap water at a temperature of 15°C. Fish were fed on commercial fish food, and acclimatized to laboratory conditions for a minimum of one week before the experiments were performed.

Experimental Procedure:

The experiment was divided into five groups for each pesticide, one control and four treated. Each group consists of ten fish in 80 liter aquaria. The four treated groups were exposed to the following concentrations; 0.01, 0.1, 0.5 and 1.0 ppm for CuSO₄; 0.0001, 0.001, 0.01 and 0.1 ppm for both ML and PQ; for 96 hours. After exposure, five fish

from each dose and the control group were anaesthetized with 150 mg/l tricaine methanesulfonate (MS 222) and dissected. Blood, taken from the ventral aorta, was collected in 600 µl Microtainer®, plasma separation tubes containing lithium heparin for enzyme detection. One drop of fresh blood was diluted with 10.0 ml of phosphate buffered saline (PBS, pH 7.4) for use in the comet assay. Liver and gill tissues were extracted from each fish. Approximately 0.2 g each of liver and gill were used for the comet assay. Small parts of these tissues were fixed in 10 % buffered formalin for tissue preparation while the rest was stored at – 80°C.

1) Comet assay

A modified version of Olive's alkaline comet assay was used (Olive *et al.*, 1994). Approximately 0.2 g each of liver and gill were homogenized in a glass polytron and suspended in 2-3 ml phosphate buffer saline (PBS) (23.5 g NaCl, 0.7 g KCl, 0.1 g K₂HPO₄, 0.2 g NaHCO₃ and 1.1 g CaCl₂.H₂O). The cell suspension was added to 500 µl PBS and centrifuged at 6500 rpm at 4°C. The supernatant was aspirated and the pellet washed with 200 µl PBS repeated 3 times. After the third wash, the pellet was suspended in 200 µl PBS and then mixed with 600 µl of 1 % low melting point agarose (US Biological Corp, Cleveland, Ohio) at 37°C. Approximately 20,000 cells were used per comet slide. The agarose suspension was layered on custom frosted slides (Smith and

O'Neill, 1998) and placed on a cold plate to allow the agarose to solidify. Three slides were prepared for each organ. The cells were lysed by bathing the slides in alkaline lysis solution (2.5M NaCl, 0.1M EDTA, 0.01M Tris-base, 1% sodium-laroyl sarcosine, pH 10) for one hour. Slides were moved to an alkaline electrophoresis buffer (0.03M NaOH, 2mM EDTA, pH 10) for 60 minutes consisting of three 20 minute baths. The slides were electrophoresed for 25 minutes at 20 V using a Bio-Rad model 200/2.0-electrophoresis unit. After five minutes of neutralizing in distilled water, the slides were stained with 2.5 µg/ml propidium iodide for 10 minutes. A coverslip was added to each slide prior to storage and analysis.

Microscopy

A Carl Zeiss epifluorescence microscope with attached CCD 72 camera and Gen II Sys Image Intensifier (DAGE-MTI Inc.) was used to visualize the comet images. Cells were examined at 400X magnification. Individual comet images were digitized and analyzed using software provided by Dr. Peggy Olive (British Columbia Cancer Research Center, British Columbia). Each damaged cell has the appearance of a comet with a brightly fluorescent head and tail whose length and fluorescence intensity are related to the number of DNA-strand breaks induced by pesticides (Fairbairn *et al.*, 1994). Undamaged cells appear as intact nuclei (comet heads) without tails (Plate 1, Fig. 1). The comet assay

essentially reflects the displacement of fluorescence from head to the tail in damaged cells (Plate 1, Fig. 2&3). The DNA damage was quantitated by the software as a tail moment which is tail length (μm) multiplied by the relative tail DNA content (expressed as a percent of DNA in tail/head) (%).

2) Antibody Detection Protocol

Pesticides exposure

Twenty four fish for each concentration were exposed to two concentrations of CuSO_4 (0.01 and 1.0 ppm), ML and PQ (0.0001 and 0.1 ppm) by immersion in the aquaria, plus control aquarium, for 96 hours.

Immunization

a) First immunization

The control group was divided into two groups; 1) non-immunized control fish (control -ve) and 2) immunized control fish (control +ve). All pesticides-exposed groups and control +ve were immunized via intraperitoneal (i.p) with 100 $\mu\text{g/g}$ fish keyhole limpet hemocyanin (KLH) (Sigma) after exposure to the pesticides.

b) Boosting immunization

The booster dose of KLH at 100 µg/g (i.p) was given after 3 weeks following the first immunization. The booster dose was injected in each fish except control –ve group.

Sampling

Three blood samples were collected from each of three rainbow trout from the treated groups before exposure to pesticides and after exposure, 1, 2, 3, 4, 5 and 6 weeks after the first immunization. Fish were anaesthetized with MS222 when each blood sample was taken. Blood was collected from ventral aorta into heparinized vacutainer tubes. The tubes were kept at 4°C overnight then centrifuged at 5000 rpm. Serum was taken to detect antibody titer level.

Anti-IgM antibody preparation

Hybridoma cells 1.14 cell line which secrete monoclonal anti- trout IgM antibody were obtained as a gift from Dr. Norman Miller (Mississippi University). The cells were cultured with RPMI 1640 media. Supernatant was taken every two days, filtered and kept at –70°C until use. The supernatant was used as 100 % concentration of anti-IgM antibody.

ELIZA

Levels of humoral antibodies to KLH were measured in indirect ELIZA technique.

Procedures:

One hundred μl of 100 μg KLH/ml is added to each of the wells of polystyrene microtitre plate (Nunc II Immunoplate, Nunc, Denmark). Each plate was incubated for one hour at 37°C. Wells were blocked with 200 μl 2% non-fat dry milk in phosphate buffered saline (PBS) immediately after coating the plate, each unit was maintained for one hour at room temperature (RT). After which wells were then washed five times with PBS with 0.05% Tween 20 (PBS-Tween 20). Subsequently, 100 μl of trout plasma diluted 1 : 100 in 2% non-fat dry milk with 0.5 % Tween 20 was added in each well. Eight serial dilutions were prepared from each fish (the dilutions were done before adding to the plate). Each plate was incubated for one hour at RT then washed five times with PBS-Tween 20. After which 100 μl of mouse monoclonal anti- rainbow trout IgM antibody, diluted 1 : 100 in 2% non-fat dry milk with 0.5 % Tween 20, was added to each well. The prepared plate was incubated for one hour at RT then washed (five times) with PBS with PBS-Tween 20. One hundred μl of rabbit anti-mouse IgG antibody conjugated to horseradish peroxidase, diluted 1:5000 in 2% non-fat dry milk with 0.5 % Tween 20 was then added per well. The plate was incubated for one hour at RT and then washed (ten

times) with PBS with PBS-Tween 20. After which 200 μ l 3,3,5,5-tetramethylbenzidine (TMB) (Sigma) was added per well. The reaction was stopped after 45 minutes with 50 μ l / well of 2 M H_2SO_4 . The optical density is read spectrophotometrically at 450 nm. The mean highest dilution of each group was used to express the titer of the humoral antibody levels.

3) Tissue Sections: Histopathology:

Liver and gill specimens fixed in 10 % buffered formalin from each fish were dehydrated, cleared and embedded in Paraplast using standard methods (Humason, 1972). Sections (4-6 μ m) were stained with Hematoxylin and eosin and Mallory's Trichrome for microscopic examination.

Slides were taken from binocular Zeiss Axioscope high resolution microscope (Carl Zeiss 7082, Oberkochen, Germany). Microscope was equipped with a 35 mm SLR camera; MC 80 system Digital equipment attached to the microscope. Sony 3CCD Color Video Camera model DXC-970MD Using a CMA-D2 camera Adapter. The images were imported into a Dell Optiplex GXA computer. Software for the digital system was Carl Zeiss Imaging Scientific Imaging Software and into ADOBE PHOTO SHOP 5.0 Images were stored in a ZIP Drive and then imported into a photo analyzer. All pictures saved as files. The pictures

were labeled and adjusted by ADOBE PHOTO SHOP software. After all the pictures became ready, they were saved as jpeg format, so it can be moved to Power Point program in windows software. In Power Point program, all pictures saved and the photos were taken on Kodak film (100, ASA). The Kodak film was put in another camera machine attached to the computer. All pictures were taken from the computer directly.

Liver cells were counted within a 400x field. Five 400x fields were done for each section and fifteen fields per slide. The average thickness of hepatic portal vein was measured by using a calibrated ocular micrometer.

4) Biochemical assay: blood enzymes activity

The blood collected in the 600 µl Microtainer® was left overnight at 4°C for complete coagulation after which it was centrifuged at 4000 rpm to isolate the serum. Each serum sample was analyzed at the blood chemistry laboratory at the Utah Valley Regional Medical Center (Provo, Utah) using Johnson & Johnson Vitros 950 Chemistry Analyzer, for detection of lactate dehydrogenase (LDH), glutamate-oxaloacetate transaminase (GOT), glutamate pyruvate transferase (GPT) and alkaline phosphatase (AP).

Statistics:

The tail moment from the comet assay for all tissues, hepatocyte morphometric and thickness of the outer layer of hepatic portal vein (*tunica adventia*) from liver sections and concentrations of blood enzymes were statistical analysed using non-parametric Kruskal-Wallis one-way ANOVA. Comparisons of all the treatment groups versus control were done.