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RESEARCH ARTICLE

The impact of several hydraulic fracking chemicals on Nile tilapia and evaluation of the protective effects of *Spirulina platensis*

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Abstract

Hydraulic fracturing (fracking) chemicals are used to maximize the extraction of hard-to-reach underground energy resources. Large amounts of fracking fluid could escape to the surrounding environments, including underground and surface water resources, during the chemical mixing stage of the hydraulic fracturing water cycle due to equipment failure or human error. However, the impact of pollution resulting from operational discharges is difficult to assess in aquatic ecosystems. In this study, pathological investigations, chromosomal aberrations, DNA damage, and biochemical and hematological parameters were used to evaluate the effects of such chemicals on Nile tilapia. Chromosomal aberrations are considered very sensitive genetic markers of exposure to genotoxic chemicals and are used as indicators of DNA damage. The appearance of different types of chromosomal aberrations (gaps and breaks) due to chemical exposure was significantly reduced by treatment with spirulina. Various deleterious findings in Nile tilapia, in the current study, could attributed to the presence of fracking chemicals in the aquatic environment. However, the presence of spirulina in the diet reduced the hazards of such chemicals. In addition, cytogenetic studies in the current work revealed the importance of spirulina in ameliorating the genotoxic effects of a mixture of some chemicals used in fracking.

Keywords Hydraulic fracturing · Fish pathology · Cytogenetic analysis · DNA fragmentation · Hematology · Spirulina

Introduction

The hydraulic fracturing (fracking) technique is widely used during drilling to open up fissures in rock for more efficient gas and oil production in Egypt (Egyptian Initiative for Personal Rights 2012) and worldwide (Morgan 2012). About 1 million oil/gas wells used hydraulic fracturing fractured in the USA since the application of this process in 1940. Many of these wells may existed near drinking water resources, consequently, its potential contamination by hydraulic, flowback fluids, and additives spills might took place and reached surface and groundwater.

Hydraulic fracturing fluids are mainly formed from 98–99.5% water and proppants (i.e., sand and ceramics) with 2–0.5% chemical additives. These chemical additives prevent bacterial growth and minimize friction as well as stop scale deposits in pipes and corrosion of well casing (Chen and Carter 2017). Exact chemical components of fracturing fluids remain unknown because chemicals and their concentrations are classified as confidential business information. The number of chemicals used in fracking might reach 1021 substances, of which about 250 chemicals suggested to have potential toxicity to human health (Kassotis et al. 2014; Kahrilas et al. 2015; Elliott et al. 2017). Three to 12 additive chemicals, depending on the characteristics of the water, could adopt typical fracture treatment and shale formation being fractured (Gawlik et al. 2017). About 15–30 million liters of fracking fluid is usually used for each well, of which about 150,000–600,000 L of chemical additives are included (Elliott et al.

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2017). Most of these chemicals exhibit potential environmental hazards to humans and animals, although reliable marine toxicity data are scarce for the majority of them. Many chemicals used in fracking are known to be poisonous (Morgan 2012), and others (such as formaldehyde, benzene, and naphthalene) are carcinogens (World Health Organization 2016).

Discharges of fracking fluids polluted rivers and water resources (Greg et al. 2004) and considered as a possible cause of groundwater contamination (Chen et al. 2017). Several routes of potential hazardous water contamination include deteriorating of well casings, instrument failure, surface release of fracturing fluid spills or wastewater on-site or during transporting fracking liquids, movement of hazardous chemicals from fractures to shallow aquifers, outflow from flowback pits, and unauthorized discharge of untreated wastewater into the environment (Elliott et al. 2017). Other reports connect these discharges to animal diseases, deaths, and second-generation birth anomalies (Goss 2013). Moreover, contaminants from frack chemicals to the environment are hazardous to human health (McKenzie et al. 2012). After fracking is performed, a portion of the pumped fluids flow back to the ground surface; this portion is termed the "flowback." Flowback can contain naturally occurring underground toxic materials, like arsenic, lead, and mercury; radioactive elements as radium (Bamberger and Oswald 2015; Harkness et al. 2015); and geogenic organic compounds (Luek and Gonsior 2017). Mall (2014) and Mahmoud et al. (2016) mentioned that fish and fish-related assays are important for the establishment of hazard identification and risk assessment protocols. Pathological studies on fish exposed to many chemicals have revealed several changes that are considered as biomarkers for marine pollution (Au 2004). Recently, Folkerts et al. (2017) observed different clinical changes in fish embryos exposed to flowback fluids.

Additionally, fish chromosomes are one of the most important bio-indicators of the presence of genotoxic materials in water (Praveen and Shadab 2012). Changes in some hematological parameters provide important clues about the health status of fish (Mazon et al. 2002; Tavares-Dias et al. 2002; Vutukuru et al. 2005; Višnjić-Jeftić et al. 2010).

Some plants and algae are helpful in reducing the hazards of toxic substances (De-Bashan and Bashan 2010). In humans, the use of *Spirulina platensis* improves the immune system and decreases cancer development and viral infection and its hot water extract activates innate immunity by enhancing interferon production and cytolysis in natural killer cells (Hirahashi et al. 2002). In addition, it enhances nonspecific immunity in channel catfish (*Ictalurus punctatus*) (Duncan and Klesius 1996). Hironobu et al. (2006) suggested that dietary spirulina has immune-stimulatory effects on the innate immune system in carp (*Cyprinus carpio*) and exhibits an antioxidant effect (Haque and Gilani 2005). Spirulina is extensively used for cleaning water polluted with different heavy metals, petroleum hydrocarbons, pesticides, and some estrogens from wastewater (Kurashvili et al. 2018).

Since hydraulic fracturing is widely practiced and the impact of pollution resulting from operational discharges is difficult to assess in aquatic ecosystems, we aimed to simulate, in part, changes in the water environment to determine the direct effects of some fracking chemicals on Nile tilapia. Moreover, we evaluated the pathological, cytogenetic, and hematological changes in Nile tilapia and the possible beneficial effects of *Spirulina platensis* on fish health status following fracking fluid exposure.

Materials and methods

This experiment has been presented and approved by the Department of Pathology Council, Faculty of Veterinary Medicine, Cairo University, on 7 July 2015.

Chemicals

In this study, we used nine fracking chemicals (United States House of Representatives. "US House of Representative" 2011), including ethylene glycol, glutaraldehyde grade I (25% in H_2O), ammonium persulfate, xylene (histological grade), EDTA (Sigma-Aldrich, USA), formalin (Horizon Chemical for Special Chemicals, Alexandria, Egypt), naphthalene (Egypt Economic Centre, ECC), copper sulfate (El-Gomhoria drug company, Cairo, Egypt), and commercial benzene. The doses used were determined according to the 50% lethal concentration (LC50); one-tenth of the LC50 for each chemical was used (Table 1).

Fish

In our study, we use 60, clinically healthy, two-month-old tilapia fish (*Oreochromis niloticus*), with an average body weight of 100 g ± 5 g and known health records, from a commercial fish farm. Fish were transported alive in a large plastic container containing water with 5 mg/L lidocaine and supplied with air via battery-operated aerators. Fish were kept in an aquaria containing de-chlorinated tap water, with an average temperature of 25 ± 3 °C and a pH of 7.17–8.19. It were fed on a standard pelleted fish diet, containing 32% protein (Joe Fid, Joe Trade Co., Cairo, Egypt), once per day, at a rate of 3% body weight and kept for 1 week without any treatment for acclimatization. Fish were divided into four groups for each replicate, 15 fish each, in a separate aquaria. Water in the aquaria was renewed with the calculated doses of chemicals every 10 days to maintain the same levels of chemicals during the 3-week experimental period.

Table 1 Frack chemicals with the reference LC dose, 1/10 calculated dose, and the calculated amount of each chemical per aquarium

Chemical	Role of chemical in hydraulic fluid	LC 50 mg/L (ref. dose)	Fish species	0.1 of LC 50 mg/L	Used dose/43 L aquarium (mg)	References
Amm. persulfate	Viscosity breakers	76.3	*Oncorhynchus mykiss* (rainbow trout)	7.63	328.09	Material safety data sheet from Thermo Fisher Scientific for ammonium persulfate
Gluteraldehyde	Eliminates bacteria in the water (biocides)	11.00	*Lepomis macrochirus* (bluegill)	1.1	47.30	Leung (2001)
Ethylene glycol	Prevents scale deposits in pipes and as a stabilizers	1490.00	*Lepomis macrochirus* (bluegill)	149.00	6407.00	Material safety data sheet from W.E. Greer Ltd., for ethylene glycol
EDTA	Water softeners (chelating agent)	430.00	*Pimephales promelas* (fathead minnow)	43.00	1849.00	Material safety data sheet from Santa Cruz Biotechnology, Inc., for EDTA
Copper sulfate	Eliminates bacteria and algae in the water	58.837	*Oreochromis niloticus* (Nile tilapia)	5.88	252.84	Ezeonyejiaku et al. (2011)
Naphthalyne	Carrier fluid for the active surfactant ingredients of fracking fluid	0.213	*Melanotaenia fluviatilis* (the Australian rainbow fish)	0.0213	0.9159	Material safety data sheet from Delek Refining, Ltd., for gas oil
Xylene	Solvents fluid to remove he organic plugs in the petroleum production (asphaltene)	3.30	*Oncorhynchus mykiss* (rainbow trout)	0.33	14.19	Material safety data sheet from UNSEI CHEMICAL Co., Ltd., for xylene
Commercial benzene	Increase the efficiency of transporting proppants in fracturing fluids	4.20	*Oncorhynchus mykiss* (rainbow trout)	0.42	18.06	Material safety data sheet from HESS corporation for Gasoline
Formalin	Eliminates bacteria in the water (biocides)	120.00	*Momne saxatilis* (striped bass)	12.00	516.00	Bills et al. (1993)

Spirulina

Spirulina platensis was obtained from VivaNutria GmbH, Hohenberg, Germany, and given to the corresponding groups as 0.5% of the pellet diet.

Experimental design

The 60 fish are divided into four groups of 15 fish each and designated as follows: the control group (C) fed the standard diet and kept without any treatment, the fracking chemical group (FC) (see Table 1) fed standard diet and exposed to chemicals added to the water. In addition, the fracking chemical and spirulina (FC+SP) groups were exposed to chemicals and fed a standard diet with spirulina; and the spirulina (SP) group fed standard diet containing spirulina, without chemical exposure. During the experimental study, we recorded any clinical alterations and collected deceased fish for immediate pathological examination. At the end of experimental period, we collected two blood samples from the caudal vein of fish in each group. The first sample was collected into a clean, dry tube containing 10% EDTA (Sigma-Aldrich, USA) and used for hematological studies and the other plain one for serum preparation and biochemical analysis.

Pathological examination

Fish were euthanized via decapitation and tissue samples including the gills, brain, skin, muscles, intestine, liver, kidneys, and reproductive organs were taken after 1 week and at the end of experimental period. The tissues were immediately fixed in 10% neutral buffered formalin, washed in tap water, passed through an ascending series of alcohol for dehydration, cleared with xylene, embedded in paraffin, and sectioned into 5–7-μm sections with a microtome (microTec CUT 4050, Germany). Sections were collected on glass slides, deparaffinized, stained with hematoxylin and eosin (HE) (Bancroft et al. 2012), and examined using a light microscope (Olympus BX50, Japan). Semi-quantitative analysis of lesions was done according to Lovasoa (2014) with modified score values.

Chromosomal preparation

A portion of the anterior kidney, of each fish, was taken for chromosomal preparation (Al-Sabti 1986), and kept in a solution of 0.56% KCl at 24 °C for 20–25 min, to allow proper swelling of cells. Tissue suspension was fixed in chilled Cornoy's fixative (3 parts of methanol and 1 part acetic acid) and slides were air-dried and stained for 20 min in 5% Giemsa. One hundred well-spread metaphases present in the prepared chromosomal slides were examined to detect different types of chromosomal aberrations.

Comet assay (single cell gel electrophoresis, SCGE)

A comet assay was carried out on peripheral blood lymphocytes from each fish, isolated by centrifugation (15 min, 280*g*) in a density gradient of Gradisol L (Aqua Medica, Lodz, Poland). The concentration of cells was adjusted to 2×10^5 cells/mL of RPMI 1640 medium (Sigma Chemicals, USA) without glutamine to produce a single-cell suspension. A freshly prepared suspension of cells in 0.75% low-melting-point agarose (Sigma Chemicals, USA) dissolved in phosphate-buffered saline (PBS, Sigma Chemicals, USA) was cast onto microscope slides pre-coated with 0.5% normal-melting-point agarose. Cells were then lysed for 1 h at 4 °C in buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, and 10 mM Tris at pH 10. After lysis, DNA was allowed to denature for 40 min in electrophoretic solution consisting of 300 mM NaOH and 1 mM EDTA, pH > 13. Electrophoresis was conducted at 4 °C for 30 min with an electric field strength of 0.73 V/cm (30 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 μg/mL ethidium bromide (Sigma Chemicals, USA), and covered with cover slips. The slides were examined by fluorescence microscopy at × 200 magnification (Nikon, Tokyo, Japan) using a Cohu 4910 video camera (Cohu, Inc., San Diego, CA, USA) equipped with a UV filter block consisting of an excitation filter (359 nm) and a barrier filter (461 nm). The equipment was connected to a personal computer–based image analysis system, Lucia-Comet v.4.51. To quantify DNA damage, tail length (TL) and tail moment (TM) were evaluated. TL (the length of DNA migration) is directly related to DNA fragment size; it is presented in micrometers and was estimated from the cell center. TM was calculated as the product of TL and the fraction of DNA in the comet tail (Belpaeme et al. 1998). TL was measured and specified as a class from zero to three. Class zero indicates no tail; class one indicates a TL < the diameter of the nucleus; class two indicates a TL between 1× and 2× the diameter of the nucleus; and class three indicates a TL > 2× the diameter of the nucleus.

Quantitative analysis of DNA fragmentation

Apoptotic DNA fragmentation was qualitatively analyzed by detecting the laddering pattern of nuclear DNA as described according to Ray et al. (1993). Briefly, liver tissues were homogenized, washed in PBS, and lysed in 0.5 mL of DNA extraction buffer (50 mM Tris–HCl, 10 mM EDTA. 0.5% Triton, and 100 μg/mL proteinase K, pH 8.0) overnight at 37 °C. The lysate was then incubated with 100 μg/mL DNase-free RNase for 2 h at 37 °C, followed by three extractions of an equal volume of phenol/chloroform (1:1 *v*/*v*) and a subsequent re-extraction with chloroform by centrifuging at 15,000 rpm for 5 min at 4 °C. The extracted DNA was precipitated in two volume of ice-cold 100% ethanol with 1/10

volume of 3 M sodium acetate, pH 5.2 at − 20 °C for 1 h, followed by centrifuging at 15,000 rpm for 15 min at 4 °C. After washing with 70% ethanol, the DNA pellet was air-dried and dissolved in 10 mM Tris–HCl/1 mM EDTA, pH 8.0. The DNA was then electrophoresed on 1.5% agarose gel and stained with ethidium bromide in Tris/acetate/EDTA (TAE) buffer (pH 8.5, 2 mM EDTA, and 40 mM Tris–acetate). A 100-bp DNA ladder (Invitrogen, USA) was included as a molecular size marker and DNA fragments were visualized and photographed by exposing the gels to ultraviolet transillumination.

Hematological and biochemical analysis

Hematological parameters including packed cell volume (PCV), hemoglobin concentration (Hb), erythrocyte count (RBCs), total leucocyte count (TLC), and differential leucocytic count (DLC) were done after Feldman et al. (2000). Serum samples were used for determination of the activities of alanine (ALT) and aspartate (AST) amino transferases, serum creatinine, serum total proteins, and serum albumin. The above-mentioned serum biochemical parameters were assayed using commercial diagnostic reagent kits supplied by Bio-diagnostic Company, Egypt.

Statistical analysis

Statistical analysis was performed with SPSS software (IBM SPSS Statistics Version 25, https:/www.ibm.com) using one-way analysis of variance (ANOVA) followed by Duncan's multiple comparisons tests (Duncan and Klesius 1996) to judge the differences between various groups and values of $P < 0.05$ were considered to indicate statistical significance.

Results

Clinical observations

Fish that received only chemical mixture (FC group) showed anorexia and signs of asphyxia, manifested by surfacing, increasing opercular movement, and aggregation around the aerator. This abnormal behavior was also noticed after changing the old water, at which times the fish were motionless and rested at the tank bottom, with a few fish demonstrating pale and bright whitish discoloration. In addition, three fish died after 1 week, and another three died during the last week. Moreover, the weight of these fish gradually decreased from an average of 100 g ± 5 g to be with an average of 89 g ± 1.2 g at the end of the experiment. Before death, these fish showed sluggish movement, general darkness in color, lethargy, and erected fins.

Fish treated with both chemicals and spirulina (FC+SP group) showed temporary anorexia; however, on the second day of exposure, the fish began to consume the diet with spirulina. The fish demonstrated higher activity levels than those exposed to the chemicals alone; two fish died shortly before the end of experiment, with an average weight of 98 g ± 0.3 g. The water in the aquaria of the two groups receiving spirulina with the diet showed slight greenish turbidity. The control fish (C group) and those fed the diet containing spirulina (SP group) reached an average weight of 110 g ± 6.5 g and showed no behavioral or clinical abnormalities.

Pathological findings

Gross lesions

External examination revealed the presence of pale red discoloration on the fish in aquaria containing water mixed with fracking chemicals, while the fish treated with spirulina appeared similar to the controls. Upon necropsy, an enlarged, dark-brown spleen was a constant finding in spirulina-treated fish throughout the experimental period.

Fish exposed to fracturing chemicals for 1 week showed subcapsular petechial and ecchymotic hemorrhages in the liver with distention of the gall bladder, and after 3 weeks, the liver was enlarged, pale, and friable. In the group treated with both chemicals and spirulina, the liver showed few petechial hemorrhages without hepatomegaly at the end of the experiment.

Histopathological findings

Microscopic alterations observed in fish that died naturally were more severe and pronounced than those observed in sacrificed fish. Gills, kidneys, liver, spleen, gonads, and brain tissues were the most affected tissue. Changes in branchial tissue were variable and ranged from lamellar necrosis and hyperplasia to mild lamellar edema. The branchial tissue of the fish treated only with chemicals showed lamellar edema and thrombosis in the lamellar capillaries after 1 week (Fig. 1 I and II). In the fish treated with both chemicals and spirulina, histopathological findings were far milder; however, capillary thrombi still existed (Fig. 1 III and IV). After 3 weeks, the lesions were similar to those at 1 week; lamellar hyperplasia, necrosis, and fusion of gill lamellae were observed in both groups exposed to chemicals, with or without spirulina (Fig. 1 V and VI). Control fish and those treated only with spirulina showed no obvious histopathological changes (Fig. 1 VII and VIII).

Pathological changes in the kidneys involved excretory and hemopoietic elements. Degenerative changes were found in the excretory portion of the kidneys in fish treated with chemicals. In addition, cystic dilatation of some glandular

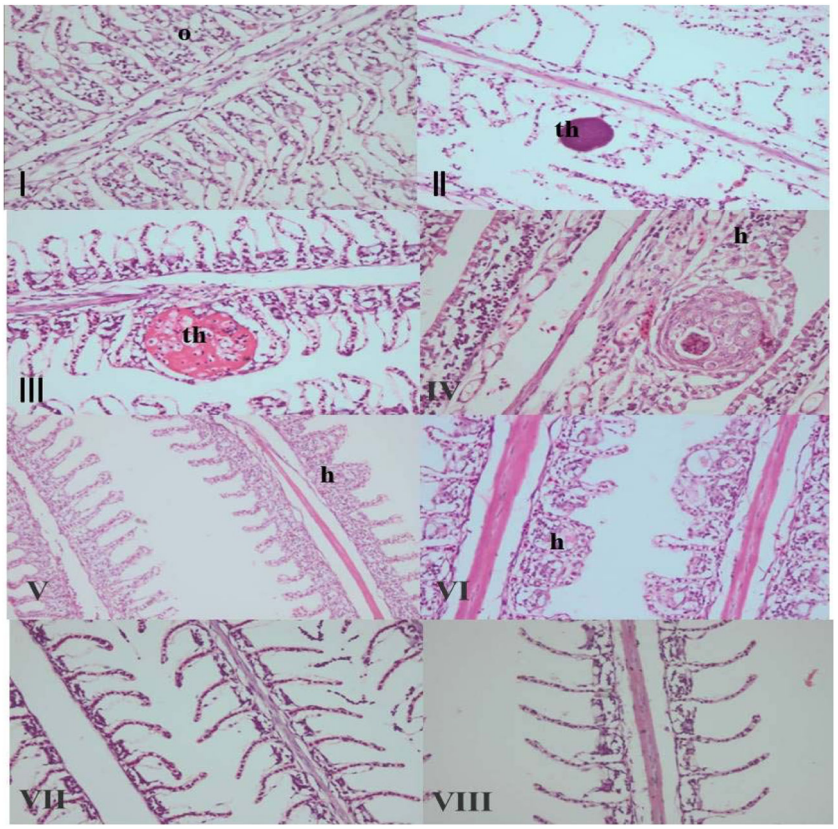

Fig. 1 Gills of *O. niloticus* fish. I, lamellar edema (o); II, thrombus formation (th) (FC group, 1 week), and III and IV (FC+SP), mild edema and thrombus (th). V (FC group), VI, (FC+SP group) after 3 weeks, focal lamellar hyperplasia (h). VII, (SP group) and VIII (control group), nearly normal branchial tissues (HE stain; I, III, IV, VI × 400; II, V, VII, VIII × 200)

tissue in the area of the stannous corpuscle was observed (Fig. 2 I). Mild to moderate degenerative changes were noticed in the epithelial lining of some renal tubules, but the glomeruli appeared normal in the excretory portion of the kidney.

The hemopoietic tissue in the kidneys and spleen showed variable degrees of necrosis, especially in the splenic subcapsular region after 1 week (Fig. 2 II). This involved sometimes both melanomacrophage centers and/or splenic ellipsoids (Fig. 2 III). After 3 weeks, vascular thrombosis (Fig. 2 IV) and hemorrhages in the spleen (Fig. 2 V) were also detected. Improvements in hemopoietic lesions were observed in the fish treated with both chemicals and spirulina, which exhibit no evidence of necrosis; however, small vascular thrombi were detected (Fig. 2 VI).

In the group exposed to chemicals, the liver exhibited congestion of the hepatoportal blood vessels after 1 week of exposure. After 3 weeks, advanced lesions involving degenerative and necrotic changes in the hepato-pancreas were a common finding. Fish exposed to both chemicals and spirulina showed mild degenerative changes in hepatocytes but had normal pancreatic tissue.

Examination of gonads revealed mild degenerative changes with reduced numbers of spermatids in the testes of the FC group male fish (Fig. 2 VII). Similarly, in the ovaries, different stages of oocyte maturation were found; the cortical alveolar stage of oocytes appeared irregular, and both vitellogenic and mature oocytes were scarce (Fig. 2 VIII). However, few abnormalities in the shape of oocytes were observed in the ovaries of FC+SP fish.

In the brain, the main findings in fish treated with chemicals only after 1 week included some scattered neuronal damage, edema of glial cells and some neurons, and focal gliosis. Mild focal and scattered neuronal necrosis, degeneration, and multifocal gliosis were noticed at the end of the experiment.

An overall summary of major pathologic lesion scores of organs was illustrated and demonstrated in graphic form (Fig. 3).

Cytogenetic analysis

The frequencies of chromosomal aberrations induced by fracking chemicals as estimated by cytogenetic analysis of fish kidneys are shown in Table 2. This analysis revealed several kinds of aberrations, such as gaps, breaks, deletions, fragments, centromeric attenuations, and polyploidy. Our results showed that the hydraulic fracturing chemicals induced a significant increase ($P < 0.05$) in the mean frequency of chromosomal aberrations compared with the vehicle control. This was especially apparent with regard to the total aberrations (6.20 ± 0.74), breaks (1.60 ± 0.25), and centromeric attenuations (1.80 ± 0.20). Gaps were also present at high frequencies

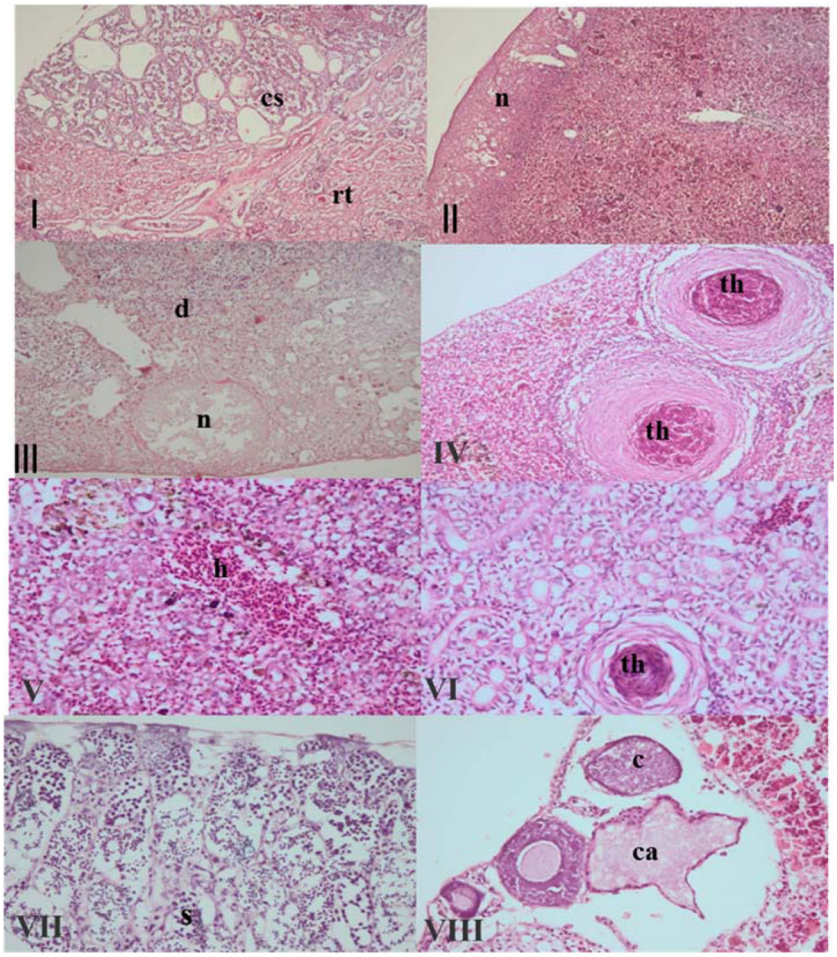

Fig. 2 I, Kidney: degenerated renal tubules (rt) and cystic dilatation in corpuscle of stannous (cs). II, spleen: diffuse area of subcapsular necrosis (n), and III, depletion of hemopoietic tissue and necrosis of melanomacrophage centers (n) (all: FC group, 1 week). IV, spleen with marked thrombosis in the blood vessels (th); and V, areas of hemorrhage (h); VI, kidney: small thrombus (th) at the peritubular capillaries (FC+SP group, 3 weeks); VII, testes: testicular tubular degeneration (s) (FC group); VIII, ovary: abnormal shape of cortical alveolar stage oocyte (ca) between perinuclear (p) and chromatin nuclear stage (c), (HE stain; I-II-III × 100; -IV-V-VI-VII x200; VIII × 400)

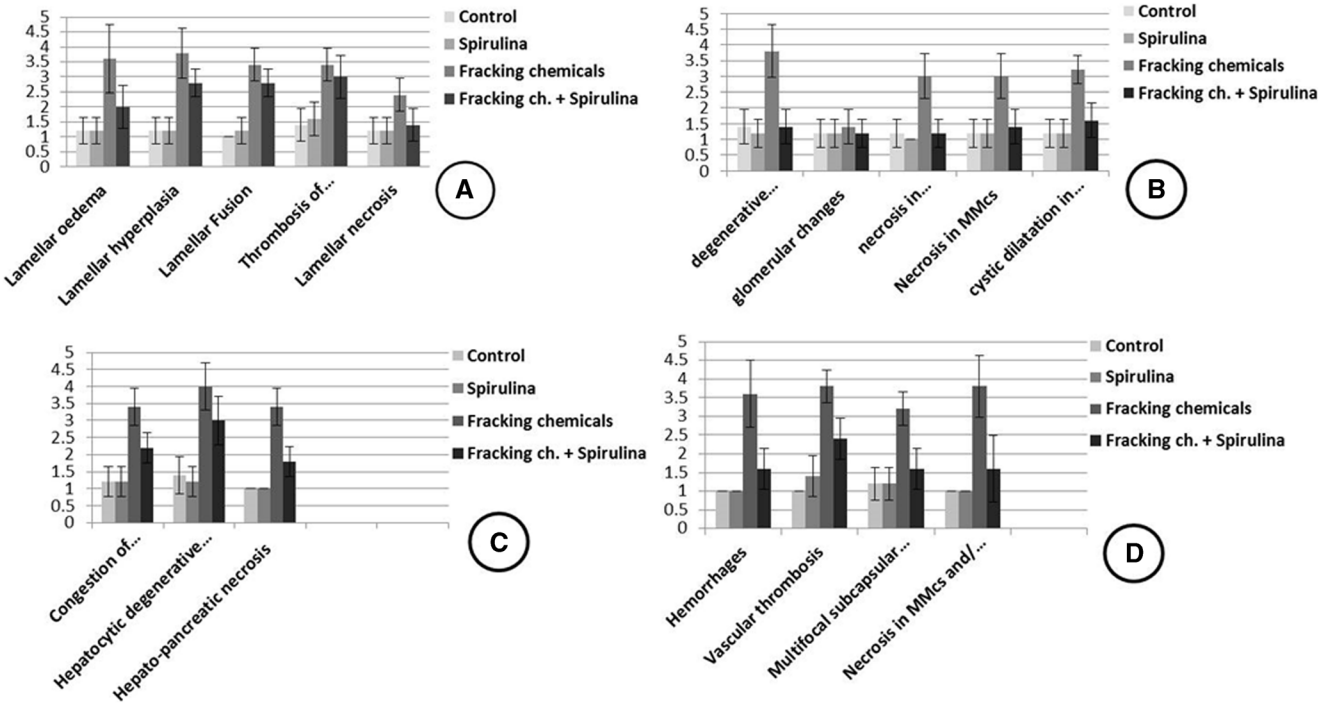

Fig. 3 Lesion scoring in different organs (means ± SD) of the examined fishes at the end of experiment: in gills (**a**), (**b**) in kidneys, in liver (**c**), in spleen (**d**). Score 1–5: 1 non/negligible, 2 mild, 3 moderate, 4 moderate to severe, 5 severe

Table 2 Chromosomal aberrations in the kidneys at the end of experimental period

Treatment group	Total aberrations without gaps	Chromosomal breaks			Centromeric attenuation	Polyploidy	Gaps
		Fragment	Deletion	Break			
Control	2.33 ± 0.96^{b}	0.33 ± 0.21^{a}	0.33 ± 0.21^{a}	0.50 ± 0.34^{b}	0.67 ± 0.21^{b}	0.0 ± 0.0	0.67 ± 0.28^{c}
Spirulina	2.40 ± 0.51^{b}	0.40 ± 0.25^{a}	0.80 ± 0.20^{a}	$0.60 \pm 0.40^{a\&b}$	0.60 ± 0.25^{b}	0.0 ± 0.0	0.80 ± 0.2^{c}
Frack chemicals	6.20 ± 0.74^{a}	0.60 ± 0.25^{a}	1.0 ± 0.32^{a}	1.60 ± 0.25^{a}	1.80 ± 0.20^{a}	1.20 ± 0.74^{a}	2.40 ± 0.25^{a}
Frack chemicals and spirulina	2.75 ± 0.48^{b}	0.25 ± 0.25^{a}	1.00 ± 0.0^{a}	0.50 ± 0.29^{b}	1.0 ± 0.41^{b}	0 ± 0.0	1.75 ± 0.25^{b}

Data were expressed as mean ± S.E.; means with different superscript letters (a, b, c) are significantly different ($P < 0.05$)

(2.40 ± 0.25) ($P < 0.01$) in the FC group compared with the control group. Spirulina supplementation slightly but non-significantly elevated the mean frequencies of chromosomal aberrations compared with vehicle. However, compared with fracking chemical treatment alone, co-administration of spirulina with chemicals significantly decreased frequencies of aberrations nearly to those in the untreated group.

Analysis of DNA fragmentation

DNA fragmentation, which is considered to be a quantitative measure of chromatin damage (McCullough et al. 2017), was evaluated in liver tissue of all fish at various times in this experiment. The results of DNA fragmentation analysis are illustrated in Figs. 4 and 5, showed significant differences between frack chemical-exposed and control groups. Fish received diet with spirulina had a significantly lower ratio of DNA fragmentation of hepatocytes than fracking chemical–exposed group.

DNA damage as assessed by comet assay

In the present study, DNA damage (Table 3) was evident in the DNA from the untreated control, spirulina-treated, fracking chemical–treated and spirulina-treated, and fracking chemical–treated groups. Treatment with fracking chemicals resulted in significantly ($P < 0.01$) higher levels DNA damage than control or spirulina-containing diet treatment. In addition, DNA damage was significantly lower ($P < 0.05$) in fish fed diets containing spirulina and exposed to fracking chemicals than in fish exposed to fracturing chemicals only (Table 3).

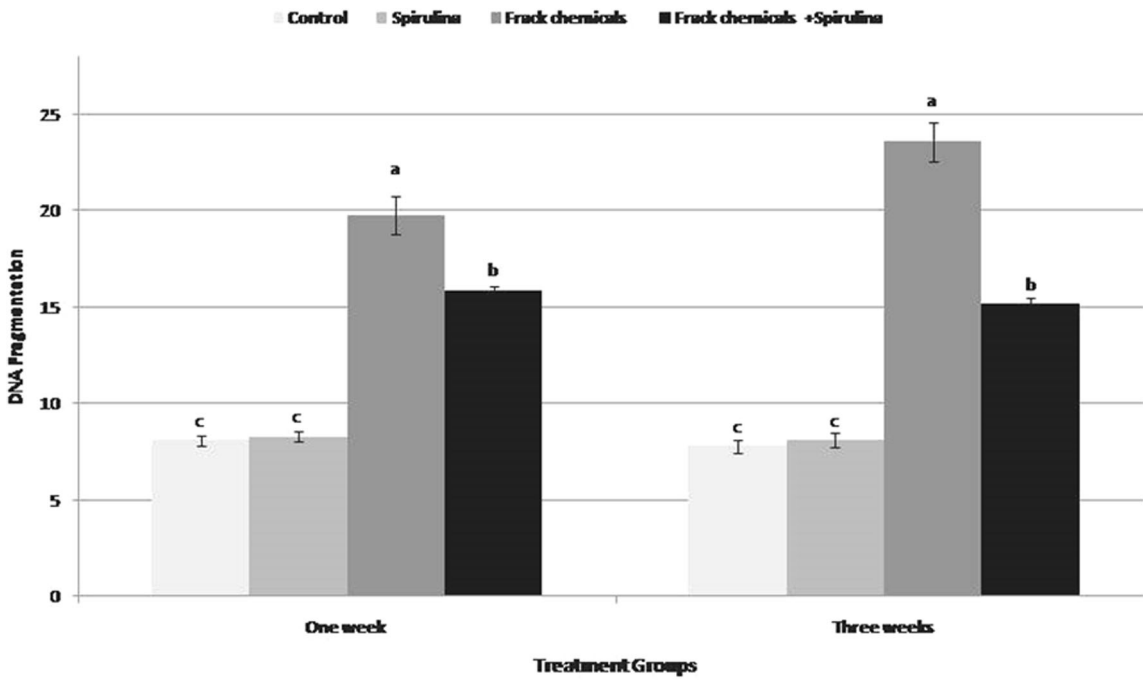

Fig. 4 DNA fragmentation of DNA extracted from the liver after 1 and 3 weeks. a, b, c Mean values in the same column with different superscript differ significantly ($P < 0.05$)

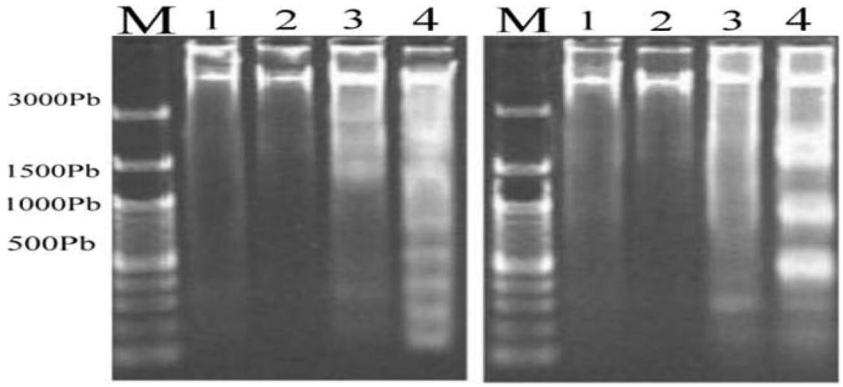

Fig. 5 DNA fragmentation detected with agarose gel of DNA extracted from the liver, after 1 and 3 weeks, by DNA gel electrophoresis laddering assay Lane 1 represents control explaining no DNA bands, 2 spirulina explaining no DNA bands, 3 spirulina + frack chemicals explaining DNA damage as more bands revealed, and Lane 4 represents fracfocus chemicals group explaining DNA damage as highest bands revealed

Hematological and biochemical studies

Mean values of packed cell volume (PCV%), hemoglobin concentration (Hb), and erythrocytic count (RBCs) were significantly decreased ($P < 0.05$) in fracking chemical–exposed fish compared with the control group (Fig. 6) with increased MCV and decreased MCHC values. Administration of spirulina to chemical-exposed fish induced improvement in erythrogram values compared with chemical-exposed fish.

Leukopenia with lymphopenia was found in fish exposed to fracking chemicals. However, significant increase in the lymphocytic count was observed in fish concurrently exposed to chemicals and spirulina in comparison with those fish exposed to chemicals only (Fig. 6). Non-significant changes were recorded in the values of neutrophilic or monocytic count of chemical-exposed fish compared with the control group.

Statistical analysis data of serum biochemical parameters ($P < 0.05$) clarified the adverse effects of fracking chemicals on hepatic and renal function in fish (Fig. 7). Fracking chemical–exposed fish exhibited marked increases in the ALT and AST activities, creatinine value, and significant decreases in serum total protein levels together with albumin.

Administration of spirulina to chemical-exposed fish decreases significantly the elevated activities of hepatic enzymes (ALT and AST), in comparison with chemical-exposed fish alone. Values of serum total proteins, albumin, globulins, and creatinine were slightly improved in-group of fish supplemented with spirulina and exposed to chemical compared with fish exposed to chemicals.

Discussion

Rapid expansion of gas production via drilling and the considerable use of hydraulic fracturing have increased gas extraction and oil resources in the USA. This increase due to the technique of fracking has elicited strong public arguments regarding possible ecological and human health hazards (Wattenberg et al. 2015). Research into the magnitude of hydraulic fracturing practices in Egypt has shown their application by two companies. One of these companies, Apache (USA), operates in the East Bahariya wells in the Western Desert. Important groundwater aquifer systems are present in the area, on which the inhabitants and the agriculture of the western oases depend. Agiba Petroleum (a joint venture of the Italian company Eni, the Russian company LUKOIL, and the International Finance Corporation (IFC)) is also using similar technology in the "Falak" and "Dorra" fields near the same region. In addition, Dana Gas (UAE) began fracking in June 2011 in the West Al Baraka-2 well near Aswan, South Egypt, which raises fears that toxic chemicals could leak into the Nile, threatening those living downstream (Egyptian Initiative for Personal Rights 2012).

In our study, relatively low mortality and morbidity occurred in fish exposed to fracturing chemicals, which may be attributed to the use of only 10% of the LC50 (1/10 LC50) of each chemical of fracking components. The emaciation observed might be a result of anorexia. Although the dose used was relatively low, the pathological changes were devastating to some fish. In practice, these chemicals are used under massive pressure, and the possibility that they could react with other naturally occurring elements must be considered. Fish deaths after some hydraulic fracturing chemical spills have been reported due to environmental exposure to fracking substances (Goss 2013). Searches for studies on the toxicity of some fracturing chemicals, such as ammonium persulfate, on fish, especially using doses similar to those in our experiment, yielded no results.

Fish are considered to be a bio-indicator species of environmental pollution, and histopathological changes in fish can indicate exposure to many toxic substances. In the present experiment, exposure of fish to several fracking chemicals induced pathological lesions in different organs, especially

Table 3 Visual score of DNA damage in the liver of different groups after 1 and 3 weeks

Treatment group	Time in weeks	Number of examined cells (*N* 300)					DNA-damaged cells (%)
		Comets*	Class**				
			0	1	2	3	
Control	One	16	284	12	4	0	5.3
	Three	15	285	11	4	0	5
Spirulina	One	17	283	12	4	1	5.7
	Three	18	282	13	5	0	6
Frack chemicals	One	74	226	24	26	24	24.7
	Three	82	218	22	31	29	27.3
Frack chemicals + spirulina	One	27	273	14	7	6	9
	Three	23	277	13	6	4	7.7

**Class 0 = no tail; 1 = tail length < diameter of nucleus; 2 = tail length between 1 and twice the diameter of nucleus; and 3 = tail length > twice the diameter of nucleus

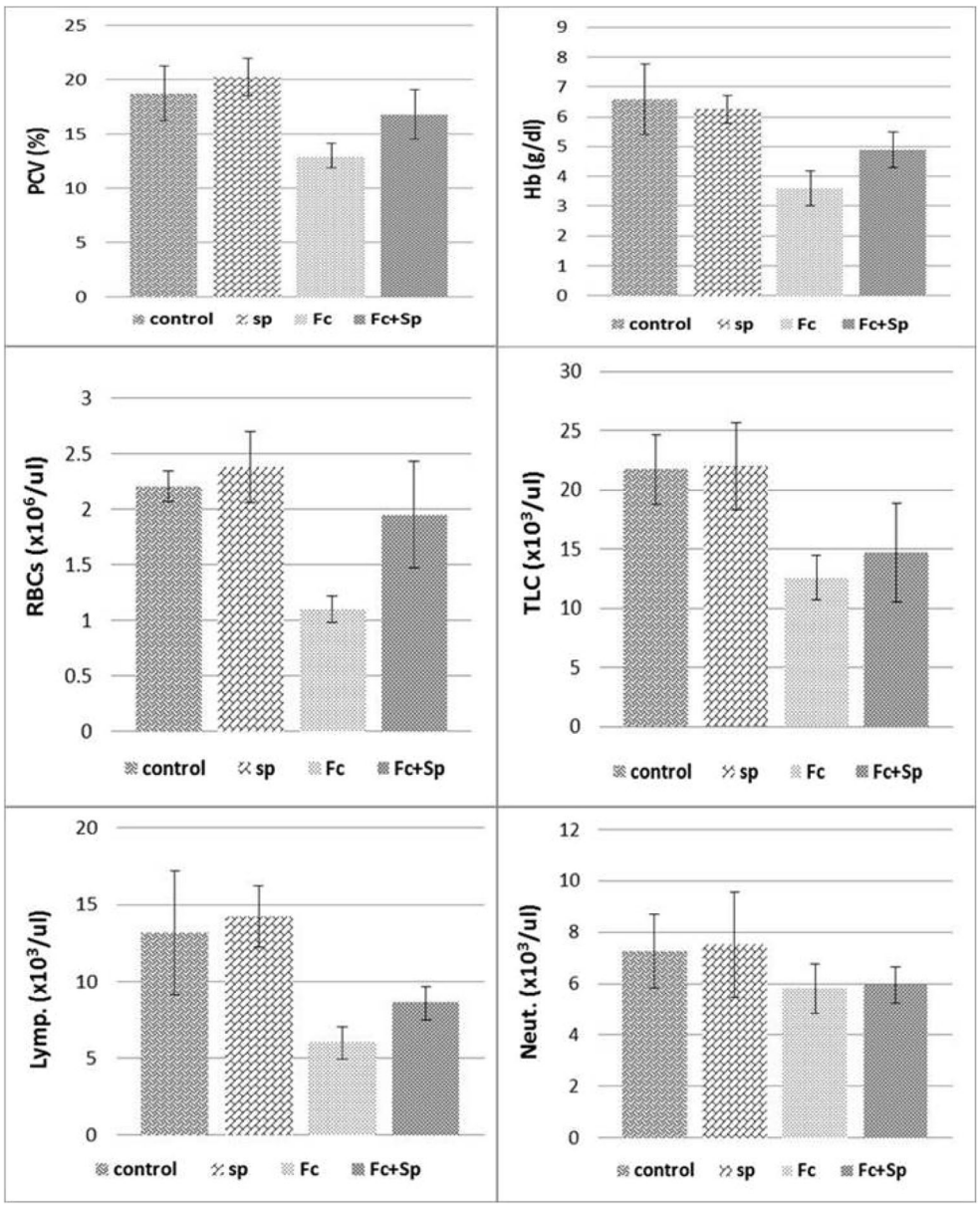

Fig. 6 Effect of spirulina and/or hydraulic fracking chemicals on hematological parameters of Nile tilapia; PCV cell volume, Hb (hemoglobin concentration), RBCs(erythrocytic count), TLC (total leukocytic count), Lymp. (lymphocytic count) and Neut. (neutrophil count). Values represent means ± SD

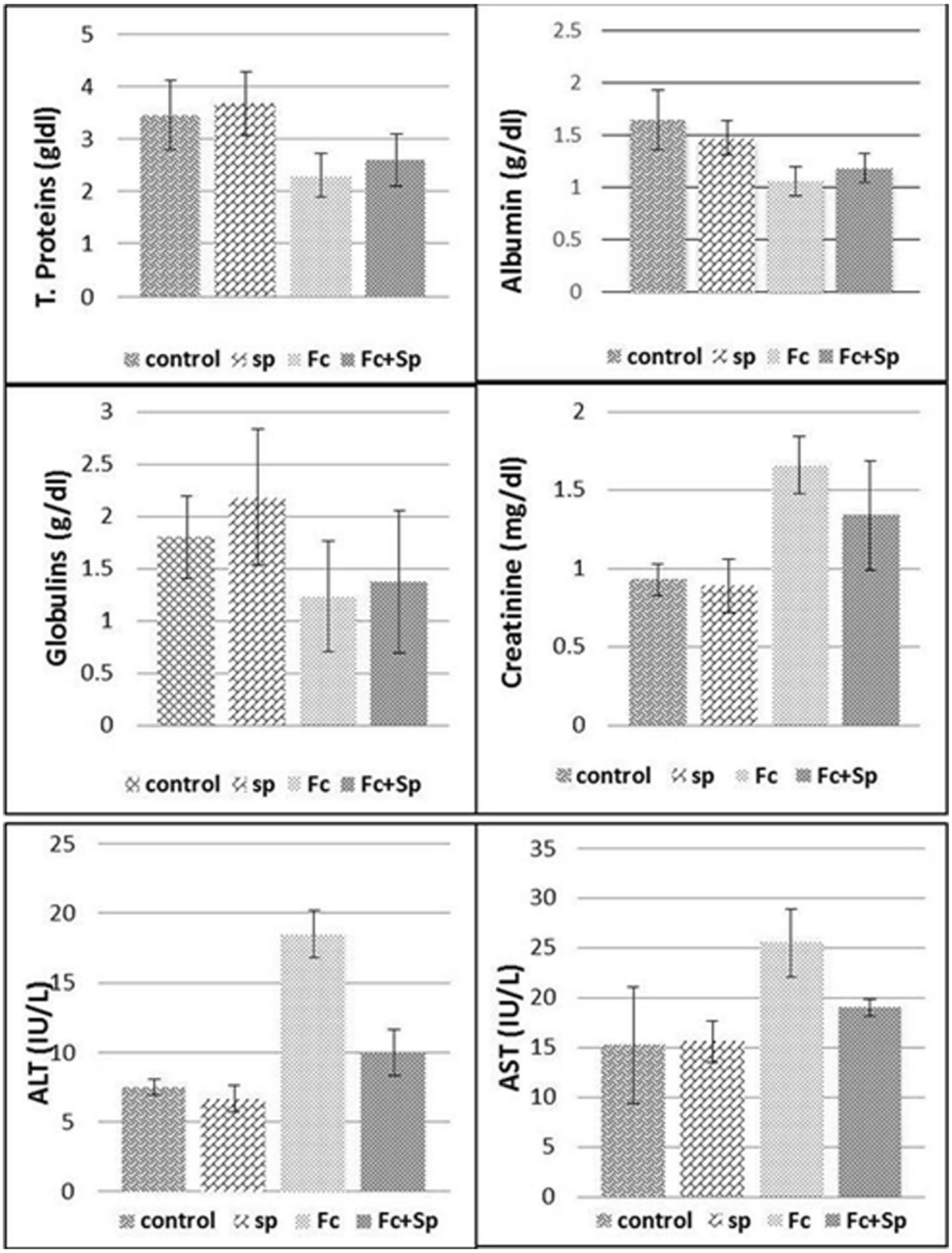

Fig. 7 Effect of spirulina and/or hydraulic fracking chemicals on some serum biochemical parameters of Nile tilapia; total proteins, albumin, globulins, creatinine, ALT (alanine amino transferase), and AST (aspartate amino transferase). Values represent means ± SD

in the gills. Flores-Lopes and Thomaz (2011) reported as that gills are useful for monitoring changes in the aquatic environment. Our findings agree with those of Papoulias and Velasco (2013) who attributed gill lesions to post-fracking deleterious effects on the branchial tissue because of the changes in water quality, including low pH and the presence of toxic metals.

Many organic compounds and toxic chemicals are supposed to have individual effects on fish or to exert effects due to the formation of new compounds. Different chemicals exhibit various hazards for animals including fish; for example, benzene, mercury, and toluene induce leukemia, brain, and kidney damage, while ethyl-benzene caused cancer (Fermanagh Fracking Awareness Network (FFAN) 2012). Similar alterations were obvious in our experiment; however, cancerous lesions were not noticed, as these require extended durations of chemical exposure. However, lamellar hyperplasia is considered to be a sign of pollution in wild fish as well as a precancerous state (Nascimento et al. 2012).

A negative impact of fracking chemicals on reproductive tissues was reported in our study, and Elliott et al. (2017) support these results recently. Moreover, androgenic, anti-androgenic, estrogenic, and anti-estrogenic chemicals have been found during analyses of water in the vicinity and/or in broad areas around gas plants (Kassotis et al. 2014). Furthermore,

hazards of long-term exposure to some hydraulic chemicals were reported in mice (Kassotis et al. 2015).

The use of spirulina in our study provided substantial protection against the toxic effects of the chemicals used. This effect manifested as reductions in the severity and distribution of pathological lesions among all organs of fish treated with both chemicals and spirulina, indicating increased tolerance of the fish to the toxic substances. In this concern, marked pathological alterations, such as necrosis and hemorrhages in hemopoietic tissue, were not observed in fish treated with both fracturing chemicals and spirulina. Abdel-Daim et al. (2013) and Shelke and Wani (2015), who revealed the protective effects of Spirulina platensis against some toxic materials, support our findings.

Fish are often used to analyze harmful cytogenetic effects of chemicals due to their capacity for bioaccumulation of toxic materials and their sensitivity to low doses of mutagens (Višnjić-Jeftić et al. 2010). Cytogenetic analysis of chromosomes can be used to assess the biological effects of any genotoxic substance on fish (Frenzilli et al. 2009). The present study demonstrated that several fracking chemicals caused chromosomal aberrations in the anterior kidneys of the fish, and the major structural changes in chromosomes were gaps, breaks, deletions, fragments, centromeric attenuations, and polyploidy. Our results are in agreement with those of Yadav and Trivedi (2009), who found that kidney cells of *Channa punctatus* exposed to copper sulfate (a fracking chemical) for 1 week showed chromatid and chromosome breaks and gaps. Another fracking chemical, naphthalene, has a powerful oxidizing effect that persists for many years (Haque and Gilani 2005). No cytogenetic effects are reported in the data sheet of the National Institute of health (NIH) (2016) based on current information regarding the toxicity and hazards of ammonium persulfate. Our results are in accordance with those of Tao et al. (2004), who found that formaldehyde exposure increases levels of cytogenetic damage in mice. The presence of chromosomal aberrations is consistent with the occurrence of DNA damage due to the accumulation of these chemicals in tissue, which inhibits electron transport into the mitochondria. Under such circumstances, marked reductions in the respiratory chain lead to the direct transfer of electrons to existing oxygen molecules, which results in the formation and accumulation of reactive oxygen species (ROS) (Xu et al. 2008). When the production of ROS exceeds the antioxidant capacity of cells, oxidative stress arises, and the resulting accumulation of these radicals causes cellular DNA damage, including aberrations in base pairs (Conklin 2000; He et al. 2017; He et al. 2018; Kassotis et al. 2018), and peroxidative damage in the kidneys (Martine et al. 2003). Our results are also in agreement with the findings of Wannee and Somphong (2007), who found that heavy metals, such as those present in fracking chemicals, caused chromosomal aberrations and DNA damage in fish and that the aberrations reached the greatest frequencies after 21 days of exposure.

Studies suggested that *Spirulina platensis* protective effect might due to its antioxidant activity (Haque and Gilani 2005). Administration of antioxidants to reduce complications associated with aging and various diseases by decreasing oxidative stress levels has been reported (Rahman 2007). In our study, administration of a diet containing spirulina to fish exposed to fracturing chemicals significantly reduced the chemical-induced chromosomal abnormalities. Spirulina contains phenolic acid, tocopherol, and β-carotene (Prahalathan et al. 2006) as well as phycocyanin and polysaccharides (Ray et al. 2007), which are known to exhibit antioxidant properties and to neutralize ROS. Our findings are in agreement with those of Premkumar et al. (2001), who reported antioxidant activity of spirulina under different oxidative conditions associated with tissue damage. Comet assays can be used to detect DNA damage in aquatic animal cells after exposure to physical or chemical irritants (Lee and Steinert 2003; De-Andrade et al. 2004). Our study showed statistically significant increases in nuclear damage by means of a comet assay after 1 and 3 weeks of exposure to fracking chemicals. The damage levels also tended to increase with the exposure time; similarly to *Eigenmannia virescens* exposed to benzene (Buecker et al. 2006). Benzene and its metabolites can prompt clastogenic and aneugenic effects in animal cells, generating micronuclei, chromosomal aberrations, sister-chromatid exchanges, and DNA strand breaks (Whysner et al. 2004). Furthermore, Buecker et al. (2012) found that the DNA strand breaks observed in a comet assay began within 48 h for 10 ppm and within 24 h for 25 ppm benzene, persisting until 96 h. They suggested that benzene induced mild irreparable damage in cells at a concentration of 10 ppm, whereas at 25 ppm, obvious genotoxic effects occurred based on the increases in comets.

A time- and dose-dependent increase in DNA damage was observed, with significant differences among various types of chemicals and physical materials, followed by a reduction at the end of the exposure, which could be attributed to DNA repair (Frenzilli and Lyons 2013).

It is worth mentioning that the present pathological and cytogenetic data were difficult to verify with previous reports, since few reports describe the toxic effects of exposure to some of these chemicals via multiple routes in humans and animals National Institute of health (NIH) (2016). In addition, the possible interactions of such chemicals under high pressures with natural toxic elements present in the earth are rather complex.

Reduction of hematologic values with increased MCV and decreased MCHC denotes the developing of macrocytic hypochromic anemia. This anemia may be due to erythropoietic

and osmoregulatory dysfunction or to an increased rate of erythrocyte destruction in hematopoietic organs (Yesudass et al. 2014). It may be due also to the oxidative effects of copper sulfate and formalin (fracturing chemicals) on erythrocytes (Vutukuru et al. 2005). Depletion of hematopoietic tissue in kidneys and spleen confirms these findings found during histopathological examination. Our results are consistent with Singh et al. (2008), who reported that copper induced significant decreases in Hb, RBCs, and PCV values of fish. It agreed also with Srivastava et al. (2009), who found significant decreases in RBCs, Hb, PCV, MCHC, and MCH values in catfish (*Heteropneustes fossilis*) exposed to formalin. The observed leukopenia with lymphopenia reflects the immunosuppressive effect of hydraulic fracking chemicals (Mazon et al. 2002). Tavares-Dias et al. (2002) reported the occurrence of lymphopenia in *Piaractus mesopotamicus* fish exposed to copper.

Statistical analysis of serum biochemical parameters clarified the adverse effects of hydraulic fracking chemicals on hepatic and renal function of fish. Fish exposed to fracturing chemicals exhibited marked increase in ALT and AST activities and creatinine value, with significant decreases in serum total proteins and albumin concentration. Increased activities of hepatotoxic biomarker enzymes, ALT and AST, are correlated with leakage of these specific enzymes from damaged hepatocytes into the blood, reflecting its disruption via fracking chemicals. Our results are in accordance with Girish and Bijoy (2014) who recorded marked increases in the activity of the ALT and AST in fish exposed to copper. Increase of creatinine level may referred to renal damage induced by the chemical as evidenced by histopathological finding in our study. The recorded hypoproteinemia reflects the hepato-renal dysfunction induced by fracking chemicals. Roja et al. (2013) found significant hypoproteinemia in common carp exposed to a sub-lethal concentration of copper sulfate (2 mg/L). Co-administration of spirulina to fish exposed to fracking chemicals reduced the altered hematological and biochemical parameters in comparison with values of fish exposed to chemicals alone. These may be due to the presence of active components in spirulina, B-carotene, linolenic acid, vitamins (C and E), and selenium, which provoke the activity of free radical scavenging processes (Adel et al. 2016).

Conclusion

The results of the present work indicate that some hydraulic fracturing chemicals have the potential to induce irreversible pathological and cytogenetic changes in fish. Previous studies on some of these chemicals have shown minor changes; therefore, the end metabolic products of chemicals mixture could lead to the development of serious changes in aquatic animals and possibly humans. In addition, blood examination can provide clues to evaluate possible chemical pollutants in specific region. Moreover, this study supports the disadvantages of the use of such chemicals, showing evidence that they exert harmful toxic and genotoxic effects in aquatic animals and most likely in humans. In addition, the results suggest that *Spirulina platensis* has the potential to repair and/or reduce pathological lesions and chromosomal aberrations and to decrease DNA damage in fish. The use of spirulina, as an agent to reduce environmental hazards in areas where some fracking chemicals are used, has to be considered.

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References

Abdel-Daim, M.M.; Abuzead, S.M.M.; Halawa, S.M. (2013). Protective role of *Spirulina platensis* against acute deltamethrin-induced toxicity in rats. PLoS One Sept. 9; 8(9):e72991. doi: https://doi.org/10.1371/journal.pone.0072991

Adel M, Yeganeh S, Dadar M, Sakai M, Dawood MA (2016) Effects of dietary *Spirulina platensis* on growth performance, humoral and mucosal immune responses and disease resistance in juvenile great sturgeon (Huso Linnaeus, 1754). Fish Shellfish Immunol 56:436–444

Al-Sabti K (1986) Clastogenic effect of five carcinogenic mutagenic chemicals on the cells of the common crab. Comp Biochem Physiol 8:5–9

Au DWT (2004) The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Mar Pollut Bull 48:817–834

Bamberger M, Oswald RE (2015) Long-term impacts of unconventional drilling operations on human and animal health. J Environ Sci Health A Tox Hazard Subst Environ Eng 50(5):447–459

Bancroft, J.D.; Suyarna, K.; Layton, C. (2012) Bancroft's theory and practice of histological techniques. 7th ed. E book ISBN: 978-0-7020-5032-9

Belpaeme K, Cooreman K, Kirsch–Volders M (1998) Development and validation of the in vivo alkaline comet assay for detecting genomic damage in marine flatfish. Mutat Res Genet Toxicol 415:167–184

Bills TD, Marking LL, Howe GE (1993) Sensitivity of juvenile striped bass to chemicals used in aquaculture. In: Resour.Publ, vol 192. Fish Wildl. Serv., U.S.D.I., Washington, DC, p 11

Buecker A, Carvalho W, Alves-Gomes JA (2006) Evaluation of mutagenicity and genotoxicity in Eigenmannia virescens (Teleostei, Gymnotiformes) exposed to benzene. Acta Amaz 36:357–364

Buecker A, Carvalho MS, Conceição MB, Alves-Gomes JA (2012) Micronucleus test and comet assay in erythrocytes of the Amazonian electric fish Apteronotus bonapartii exposed to benzene. J Braz Soc Ecotoxicol 7(1):65–73

Chen H, Carter KE (2017) Characterization of the chemicals used in hydraulic fracturing fluids for wells located in the Marcellus Shale Play. J Environ Manag 200:312–324

Chen SS, Sun Y, Tsang DCW, Graham NJD, Ok YS, Feng Y, Li XD (2017) Insights into the subsurface transport of As (V) and Se (VI) in produced water from hydraulic fracturing using soil samples from Qingshankou Formation, Songliao Basin, China. Environ Pollut 223:449–456

Conklin KA (2000) Dietary antioxidants during cancer chemotherapy: impact on chemotherapeutic effectiveness and development of side effects. Nutr Cancer 37(1):1–18

De-Andrade VM, De-Freitas TR, Da Silva J (2004) Comet assay using mullet (Mugil sp.) and sea catfish (Netuma sp.) erythrocytes for the detection of genotoxic pollutants in aquatic environment. Mutat Res 560:57–67

De-Bashan LE, Bashan Y (2010) Immobilized microalgae for removing pollutants: review of practical aspects. Bioresour Technol 101: 1611–1627

Duncan PL, Klesius PH (1996) Effects of feeding spirulina on specific and nonspecific immune responses of channel catfish. J Aquat Anim Hlth 8(4):308–313

Egyptian Initiative for Personal Rights (2012) Oil companies in Egypt use controversial technology banned in a number of countries-EIPR warns: gas extraction using hydraulic fracturing threatens Egypt's water resources. http://eipr.org/en/pressrelease/2012/09/19/1492

Elliott EG, Ettinger AS, Leaderer BP, Bracken M, Deziel NC (2017) A systemic evaluation of chemicals in hydraulic-fracturing fluids and wastewater for reproductive and developmental toxicity. J Expo Sci Environ Epidemiol 27(1):90–99. https://doi.org/10.1038/jes.2015.81

Ezeonyejiaku CD, Obiakor MO, Ezenwelu CO (2011) Toxicity of copper sulphate and behavioral locomotor response of tilapia (Oreochromis niloticus) and catfish (Clarias gariepinus) species. Online J Anim Feed Res 1:130–134

Feldman BF, Zinkl JG, Jain NC (2000) Schalm's veterinary hematology, 5th edn. Lea and Febiger, Philadelphia, U.S.A., p 1120

Fermanagh Fracking Awareness Network (FFAN) (2012). http://www.frackaware.com/wordpress/wp-content/uploads/2012/08/Fishing-Health-and-What-is-Fracking.pdf

Flores-Lopes F, Thomaz AT (2011) Histopathologic alterations observed in fish gills as a tool in environmental monitoring. Braz J Biol 71(1): 179–188

Folkerts EJ, Blewetta TA, He YH, Goss GG (2017) Cardio-respirometry disruption in zebrafish (*Danio rerio*) embryos exposed to hydraulic fracturing flow back and produced water. Environ Pollut 231(2): 1477–1487

Frenzilli G, Lyons BP (2013) The comet assay in marine animals. Methods Mol Biol 1044:363–372

Frenzilli G, Nigro M, Lyons BP (2009) The comet assay for the evaluation of genotoxic impact in aquatic environments. Mutat Res 681: 80–92

Gawlik BM, Tavazzi S, Mariani G, Skejo H, Comero S (2017) Analysis of chemical constituents and additives in hydraulic fracturing waters. Joint research Center technical report, Publications Office of the European Union, Luxembourg

Girish KB, Bijoy NS (2014) Copper toxicity: hematological and histopathological changes and prophylactic role of vitamin C in the fish, Anabas testudineus (Bloch, 1792). J Zoo Stud 1(3):4–13

Goss S (2013) Fracking fluids spill caused Kentucky fish kill. http://www.ewg.org/enviroblog/2013/09/fracking-fluids-spill-caused-kentucky-fish-kill-

Greg R, Robert H, Norman J (2004) Unlocking the Monterey Shale potential at Elk Hills: a case study. In SPE international thermal operations and heavy oil symposium and western regional meeting.16-18 March, Bakersfield, California, USA.

Haque SE, Gilani KM (2005) Effect of ambroxol, spirulina and vitamin-E in naphthalyne induced cataract in female rats. Indian J Physiol Pharmacol 49(1):57–64

Harkness JS, Dwyer GS, Warner NR, Parker KM, Mitch WA, Vengosh A (2015) Iodide, bromide, and ammonium in hydraulic fracturing and oil and gas wastewaters: environmental implications. Environ Sci Technol 49(3):1955–1963. https://doi.org/10.1021/es504654n

He Y, Folkerts EJ, Zhang Y, Martin JW, Alessi DS, Goss GG (2017) Effects on biotransformation, oxidative stress, and endocrine disruption in rainbow trout (Oncorhynchus mykiss) exposed to hydraulic fracturing flowback and produced water. Environ Sci Technol 51(2):940–947

He Y, Sun C, Zhang Y, Folkerts EJ, Martin JW, Goss GG (2018) Developmental toxicity of the organic fraction from hydraulic fracturing flowback and produced waters to early life stages of zebrafish (Danio rerio). Environ Sci Technol 52(6):3820–3830

Hirahashi T, Matoto M, Hazeki K, Saeki Y, Ui M, Seya T (2002) Activation of the human innate immune system by spirulina: augmentation of interferon production and NK cytotoxicity by oral administration of hot water extract of Spirulina platensis. Inter Immuno-pharmacol 2:423–434

Hironobu W, Kazuki O, Asmi C, Tassakka T, Masahiro S (2006) Immuno-stimulant effects of dietary *Spirulina platensis* on carp. Cyprinus carpio Aquac 258:157–163

Kahrilas GA, Blotevogel J, Stewart PS, Thomas-Borch T (2015) Biocides in hydraulic fracturing fluids: a critical review of their usage, mobility, degradation, and toxicity. Environ Sci Technol 49:16–32. https://doi.org/10.1021/es503724k

Kassotis CD, Tillitt DE, Davis JW, Hormann AM, Nagel SC (2014) Estrogen and androgen receptor activities of hydraulic fracturing chemicals, surface and ground water in a drilling-dense region. Endocrinology. 155(3):897–907. https://doi.org/10.1210/en.2013-1697

Kassotis CD, Klemp KC, Vu D, C; Lin C-H, Meng C-X et al (2015) Endocrine-disrupting activity of hydraulic fracturing chemicals and adverse health outcomes after prenatal exposure in male mice. Endocrinology. 156(12):4458–4473. https://doi.org/10.1210/en.2015-1375

Kassotis CD, Nagel SC, Stapleton MH (2018) Unconventional oil and gas chemicals and wastewater-impacted water samples promote adipogenesis via PPARγ-dependent and independent mechanisms in 3T3–L1 cells. Sci Total Environ Nov 01(640-641):1601–1610. https://doi.org/10.1016/j.scitotenv.2018.05.030

Kurashvili M, Varazi T, Khatisashvili G, Gigolashvili G, Adamia G, Pruidze M, Gordeziani M, Chokheli L, Japharashvili S, Khuskivadze N (2018) Blue-green alga spirulina as a tool against water pollution by 1,1′-(2,2,2-trichloroethane-1,1-diyl)bis(4-chlorobenzene) (DDT). Annals Agrarian Sc 16(4):405–409. https://doi.org/10.1016/j.aasci.2018.07.005

Lee RF, Steinert S (2003) Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutat Res 544:43–64

Leung HW (2001) Ecotoxicology of glutaraldehyde: review of environmental fate and effects studies. Ecotoxicol Environ Saf 49(1):26–39

Lovasoa C (2014) Study of treated and untreated oil based drilling waste using a biomarker approach: gill and liver histopathology in Atlantic salmon (*Salmo salar*). MSc. Environmental Technology/Offshore Environmental Engineering. In: Faculty of Science and Technology. University of Stavanger, Norway

Luek JL, Gonsior M (2017) A review of organic compounds in hydraulic fracturing fluids and wastewaters. *Water Res* 123:536–548. https://doi.org/10.1016/j.watres.2017.07.012

Mahmoud MA, Abdelsalam M, Mahdy OA, El Miniawy HMF, Ahmed ZA et al (2016) Infectious bacterial pathogens, parasites and pathological correlations of sewage pollution as an important threat to farmed fishes in Egypt. Environ Pollut 219:939–948

Mall, A. (2014). Halliburton takes 5 days to provide chemical information at Ohio fracking explosion that killed 70,000 fish. NRDC. https://www.nrdc.org/experts/amy-mall/halliburton-takes-5-days-provide-chemical-information-ohio-fracking-explosion.

Martine GR, Loureiro AP, Marques SA, Miyamoto S, Yamaquchi LF (2003) In: Oxidative and alkylating damage in DNA. Mutation Res./Rev. Mutat. Res., 544, (2-3): 115- 127

Mazon AF, Monteiro EAS, Pinheiro GHD, Fernandes MN (2002) Hematological and physiological changes induced by short-term

exposure to copper in the freshwater fish. Prochilodusscrofa Braz Biol 62:621–631
McCullough SD, On DM, Bowers EC (2017) Using chromatin immunoprecipitation in toxicology: a step-by-step guide to increasing efficiency, reducing variability, and expanding applications. Curr Protoc Toxicol 2; 72:3.14.1-3.14.28. https://doi.org/10.1002/cptx.22
McKenzie LM, Witter RZ, Newman LS, Adgate JL (2012) Human health risk assessment of air emissions from development of unconventional natural gas resources. Sci Total Environ 424:79–87
Morgan RD (2012) What the frack? An empirical analysis of the effect of regulation on hydraulic fracturing. Quinnipiac Hlth Law J 16(1):77–114
Nascimento AA, Arau'jo FG, Gomes ID, Mendes RMM, Sales A (2012) Fish gills alterations as potential biomarkers of environmental quality in a eutrophized tropical river in South-Eastern Brazil. Anat Histol Embryol 41:209–216
National Institute of health (NIH) (2016) https://pubchem.ncbi.nlm.nih.gov/compound/Ammonium_persulfate#section=Non-Human-Toxicity-Values
Papoulias DM, Velasco A (2013) Histopathological analysis of fish from Acorn Fork Creek, Kentucky, exposed to hydraulic fracturing fluid releases. Southeast Nat (12):92–111
Prahalathan C, Selvakumar E, Varalakshmi P (2006) Lipoic acid modulates Adriamycin induced testicular toxicity. Reprod Toxicol 21:54–59
Praveen N, Shadab GA (2012) Cytogenetic evaluation of cadmium chloride on Channa punctatus. J Environ Biol 33:663–666
Premkumar K, Pachiappan A, Abraham SK, Santhiya ST, Gopinata PM, Ramesh A (2001) Effect of Spirulina fusiformis on cyclophosphamide and mitomycin-C induced genotoxicity and oxidative stress in mice. Fitoterapia 72:906–911
Rahman K (2007) Studies on free radicals, antioxidants, and co-factors. Clinic Inter Aging 2(2):219–236
Ray J, Peterson DA, Schinstine M, Gage FH (1993) Proliferation, differentiation, and long-term culture of primary hippocampal neurons. Proc Natl Acad Sci 90:3602–3606
Ray S, Roy K, Sengupta C (2007) Evaluation of protective effects of water extract of spirulina platensis (blue green algae) on cisplatin-induced lipid peroxidation. Indian J Pharm Sci 69:378–383
Roja SJ, Abbas B, Maryam G, Seyed MV, Maryam T (2013) Impact of copper sulphate on hematological and some biochemical parameters of common Carp (Cyprinus carpio L., 1758) in different pH. World J. Fish & Marine Sc 5(5):486–491
Shelke AD, Wani GP (2015) Protective effect of dietary supplementation of *Spirulina platensis* on improvement of growth parameters in mercuric chloride exposed fish. Int J of Life Science (A3):38–41
Singh D, Nath K, Trivedi SP, Sharma YK (2008) Impact of copper on hematological profile of freshwater fish, Channa punctatus. J Environ Biol 29:253–257
Srivastava S, Gupta A, Chaturvedi S, Abhinav A, Singh R (2009) Effects of short-term therapeutic bath of malachite green and formalin on certain hematological parameters of a freshwater catfish, (*Heteropneustes fossilis*) (Bloch). J Exp Zool 12(1):79–83
Tao XY, Yu SY, Kang L, Huang HX, Wei AY (2004) Study on the genetic damage in mice induced by the volatile organic compounds of decoration materials. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing ZaZhi, 22: 194-196
Tavares-Dias M, Martins ML, Schalch SH, Onaka CIF, Quintana JRE et al (2002) Hematologic and histopathologic changes in pacu (Piaractusmes opotamicus) Holmberg, 1887 (Osteichthyes, Characidae) treated with copper sulfate (CuSO4). Acta Scientiarum Maringá 24(2):547–554
United States House of Representatives. "US House of Representative" (2011). Chemicals used in hydraulic fracturing. Washington, DC: Committee on Energy and Commerce APRIL 2011
Višnjić-Jeftić Z, Jarić I, Jovanovic L et al (2010) Heavy metal and trace element accumulation in muscle, liver and gills of the Pontic shad (*Alosa immaculata Bennet* 1835) from the Danube River (Serbia). Microchem J 95(2):341–344
Vutukuru S, Suma C, Madhavi K, Juveria S, Pauleena J, Raoand J, Anjaneyulu Y (2005) Studies on the development of potential biomarkers for rapid assessment of copper toxicity to freshwater fish using Esomusdanricus as model. Int J Environ Res Public Health 2(1):63–73
Wannee JK, Somphong S (2007) Efficacy of ascorbic acid reducing water bone copper toxicity in butterfish (Poronotus triacanthus). J Biol Sci 7:620–625
Wattenberg EV, Bielicki JM, Suchomel AE, Sweet JT, Vold EM, Ramachandran G (2015) Assessment of the acute and chronic health hazards of hydraulic fracturing fluids. J Occup Environ Hyg 12(9): 611–624. https://doi.org/10.1080/15459624.2015.1029612
Whysner J, Reddy MV, Ross PM, Mohan M, Lax EA (2004) Genotoxicity of benzene and its metabolites. Mutat Res 566:99–130
World Health Organization (2016) http://monographs.iarc.fr/ENG/Classification/index.php
Xu J, Lian LJ, Wu C, Wang XF, Fu WY, Xu LH (2008) Lead induces oxidative stress, DNA damage and alteration of p53, Bax and Bcl-2 expressions in mice. Food Chem Toxicol 46(5):1488–1494
Yadav KK, Trivedi SP (2009) Chromosomal aberrations in a fish Channa punctatus after in vivo exposure to three heavy metals. Mutation Res Gen Toxicol Environ Mut 1:7–12
Yesudass T, Sekar J, Muthusamy P (2014) Effect of copper toxicity on hematological parameters to fresh water fish Cyprinus Carpio (Common Carp). J Environ Sci Toxicolo Food Technology 8:50–60