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Effects of Short-Term Exposure to Crude-Oil on the Ultrastructure of Hepatocytes and Observations on Growth, Protein, RNA, DNA Concentration and their Ratios in Flatfish Flounder (*Platichthys flesus*)

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Abstract: The crude oil-induced growth rate, protein synthesis, RNA : DNA ratio indices and electron microscopical investigations on liver and on other tissues have been investigated in the flounder, *Platichthysflesus*. The liver registering greater sensitivity to crude oil exposure. Different ultrastructural changes in hepatocytes included: appearance of large lysosomal vacuoles; distorted and swollen mitochondria with affected cristae; endoplasmic reticulum segments with dilation; loss of ribosomes, edema progressing to the state of cytoplasmic chaos and the cell rupture. The cell membrane of affected hepatocytes exhibited consistent irregular appearance, with a small series of fold and hepatocytes themselves becoming irregular; however, necrotic hepatocytes were seldom observed. The crude oil further has an obvious effect on growth rates and protein synthesis. The synthesis rate was observed in the order of decreasing sensitivity to crude oil as: liver > whole body > white muscles. The observations on RNA concentration, RNA/DNA ratio and the protein concentration were significantly affected and ranked as: whole body > liver > white muscles. The protein concentration have the same pattern as of RNA concentration. It was concluded that the present exposure of crude oil, based on various parameters affected in various ways to consumable fishes and hence considered serious for human health.

Key words: *P. flesus* · Crude Oil · Histopathology · Growth · RNA/DNA Ratios

INTRODUCTION

The crude oil (petroleum), predominantly hydrocarbons, comprising long chains of carbons and hydrogen [1] is a widespread pollutant entering as waste from land or water and further, serious exposure is caused by the clean-up operations on shoreline: thus creating negative impact on the environment and seriously causing a variety of effects in marine life. Oil components, some very toxic, can accumulate in the organisms [2,3]. Studies taken earlier have indicated that oil interferes with the reproduction, development and growth rates [4] and the induction response is generally observed in liver; however, other organs and tissue are also affected [5]. Tissue specific differences are common, some sub cellular changes appear to have patterns of similarity among several species. It is often exhibited in the form of considerable anatomical alterations in response to

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contaminants [6], including effect on the immune system [7]. Consequently, it is established that fishes and marine organisms have the ability to accumulate contaminants usually occurring at very low concentrations. When toxic contaminants are waterborne, the gills and liver are the site of damage, thus making favorable materials to be assayed [8-11]. Seriously enough, it increases susceptibility to diseases as well and inhibits growth in fishes [1].

Effects on fishes due to contaminated food, heavy metals, nitrogenous compounds and pesticides are also well documented [12-15]. In comparison, studies on hydrocarbons are few [16]. Thus we find many reports with hazardous effects on fish health are found [17-20], compared to exposure to hydrocarbons and serious impairment to some organs, besides inflicting mortality [16, 21, 22]. Similarly, effects due to nitrogenous compounds [23], pesticides [24], stress[25] and due to unspecified causes [26, 27] are also recorded. All these observations point to, that pollutants enter the aquatic environment and make their way in the tissues of aquatic vertebrates [28]. In view of hazards arising out of these varied chemicals, it becomes very serious considering fishes make a predominant food among humans. The consumption of fishes together, with other aquatic animals is a rapidly growing sectors in the food industry and so a higher demand for healthy forms of fish meat and oil is increasing [29]; the fact stressing further a strict monitoring for all kinds of pollutants.

There are many parameters used to see the effect of a chemical candidate. In fishes including *P. flesus*, enzymes activity, micronuclei, CA have long been considered popular parameters to monitor genotoxicity [30, 31, 32]. Molecular methods based on nucleic acid derived indices [33, 34] have also increasingly been used as an important parameters of nutritional condition. Since, the ultrastructure of liver cells of *teleosts* respond very favorably and with a greater sensitivity, exploiting this responsiveness is still the most favoured indicator in monitoring the pollution effect on aquatic animals, more as a direct evidence [12, 14, 35].

However of late, more molecular indicators such as nucleic acids and protein based factors have been incorporated to have realistic assessment of growth [36, 37]. The reason being, variation of RNA concentration and protein synthesis can respond very quickly to the changes in feeding strategies [38, 39]. To date, growth rates of fish species have been successfully evaluated by the application of nucleic acids and protein assessments [40, 41]. Incidentally, many fish species continued to be benefited from studies on body length and body mass to estimate the rate of growth [42] and while doing so the need for ultrastructural changes of liver, response on muscle cells as well as on whole body are the parameters considered simultaneously.

In the light of above mentioned literatures, present studies were designed to integrate the histopathological conditions of liver and other observations on white muscles and whole body level. The hepatocytes are mainstay of the focus both in the normal state of liver as well as post exposure conditions to crude oil. A time and dose-related response is pursued using gavage method through various biomarker responses. A comprehensive histopathological evaluation of the hepatocytes and alterations of the damage is embarked upon. The other aim is to investigate the physiological status by focusing on the pattern of growth affected, using RNA, DNA and protein profiles and using these indices to assess the growth rate more logically. The study assumes significance in the light that there is an urgent need for baseline species-specific validation data, before the generalized application of logical estimate of the damage and suitability of fishes as food are ascertained.

MATERIALS AND METHODS

Sample Collection, Handling and Acclimatization: Samples of flounder, *Platichthys flesus* were collected from the coast of Aberdeen through local fishermen using gillnets. The fishes were transported to the Laboratory of Zoology Department, University of Aberdeen. The collection were stocked in the rectangular cement tanks of 1 x 1m size, having a gentle flow of sea water maintained at 19 °C and with dissolved oxygen of 8.0 mg/L, besides maintaining the natural photoperiod [43]. All fishes were acclimatized in these conditions for seven days. A defined number of fishes from this stock were used and distributed in defined groups utilizing different parameters. The experiments were conducted on 18 flounders with the mean initial weight of 2117.8 g (± SE) and with the mean length of 51.62 cm. throughout the experiment, oxygen saturation was maintained at 95.6% with the mean salinity, 34.11‰(± SE) and pH at 8.1 (± SE).The specimen were fed to a mixture of minced sand-eels *ad libitum* in the morning (11:30 AM). The uneaten food was withdrawn and weighed on daily basis. No sign of disease or death recorded during acclimatization and experimentation period. The environmental parameters maintained during experiments are summarized in Table 1.

Table 1: Showing the environmental conditions for acclimatization during the study of flounder *Plantichthys flesus*

Conditions	Mean±SE	Number of fishes
Temperature (°C)	13.32±0.164	18
Oxygen (O_2)	95.64±1.3	
Salinity (S%)	34.11±0.11	
pH	8.07±0.23	

Crude Oil Exposure: The crude oil procured from Aberdeen University Research Institute (AURIS) was introduced into the fishes by gavage method. The dose was fixed at 0.2 ml/kg. The specimen were left for seven days during which all the measurements and weighing done. The exposed individuals were sacrificed for requisite tissue procurement such as liver, muscles and whole body in a fixed quantity. Four fishes were saved and used as controls.

Procedure for Growth and Protein Assessment: The whole-body and protein specific growth rate were calculated for the duration of experimental period and after termination of experiment for two weeks using Ricker method [44]. The fishes were starved for 24 hrs before commencing weighing. This was to make sure that ingested food was evacuated. For protein growth calculations, W_1 and W_2 were recorded denoting the initial and final protein content of the fishes. The initial protein content was estimated for fresh weight of the fish and the protein concentration of the initial group. The growth rate determination of each group was carried out fortnightly.

To Estimate rate of Protein Synthesis K_s: To estimate the protein synthesis rates NaOH counts were multiplied by 5 to give DPM per 5 ml; divided by mg protein per 5 ml so as total protein in 5 ml samplegiving DPM per nmole Phe was obtained. To calculate nmolPhe/ml from B-Phe standard curve, hydrolysate was multiplied by 5 to be assayed as 0.1 ml of sample. To calculate DPM/ml from the scintillation counts: 2 ml of hydrolyte was assayed divided by 2 to get DPM/ml for assessment. The following equation was used to estimate the protein synthesis:

$$\text{Est } K_S = \frac{\frac{DPM}{nmol}Phe}{1400} \text{ X } \frac{100}{time(minutes)} \text{ X } 1440 \text{ (\%/day)}$$

To calculate DPM/nmolPhe for hydrolysates and free pools – used the following equation:

$K_s = S_b$ (DPM/nmolHyd)/S_a (DPM/nmol FP) X 100/time (minutes) x 1400 (%/day)

To Estimate RNA, DNA Concentration and RNA/DNA Ratio: To estimate the RNA, DNA concentration and RNA/DNA ratio, the procedure of Tong*et al*.[45] was followed. Samples of liver, white muscles and whole body were separately homogenized and processed in an ice-cold fixative (0.05 M Tris, 0.1 M NaCl, 0.01 M EDTA, pH 8.0) taken in a glass homogenizer, placed in 19 °C ice-cold water. The homogenate thus collected was divided into two lots; one, was used to determine the concentration of nucleic acids and the remaining utilized to measure protein content.

Nucleic acids were extracted and analyzed using a UV-based method [39, 45]. To begin with: free nucleotides were removed by a series of washes in cold perchloric acid ($HClO_4$). The solution was then hydrolyzed with potassium hydroxide; the hydrolysate was acidified with cold $HClO_4$ to remove RNA, from DNA and protein. The DNA was hydrolyzed and separated from the remaining protein by the addition of warm $HClO_4$. Care was taken to confirm the absence of RNase and DNase in the reagents. The nucleic acids were estimated by the absorbance of the hydrolysate at 260 nm using the extraction coefficient – A260 of a 1 µg mL–1 solution of hydrolyzed RNA or DNA as 0.3. Absorbance was measured using the instrument *GeneQuantpro* (Biochrom Ltd., UK). Protein content was analyzed with an assay following the Bradford method [46].Total RNA and DNA were extracted from liver, white muscles and whole-body samples in triplicates and quantified using the dual wavelength method [47]. The RNA and DNA concentration were expressed as µg nucleic acid per 100 mg wet weight. The calculations for RNA performed as under:

RNA (µg/ml) = 10.53 x [(abs_{260} x 3.17) – (abs_{232} x 0.75)]
RNA in sample = RNA (µg/ml) x 4.767 x 5/3.9 x 6.112

The calculations for DNA was obtained as follows:
DNA (µg/ml) = 10.53 x [(abs_{260} x 3.17) – (abs_{232} x 0.75)]
DNA is sample = DNA (µg/ml) x 5 x 5/3.9 x 6.4103

Procedure for Electron Microscopy: Electron microscopy procedure followed the Segner and Möller [48] method. Salient steps included:

Small portions of caudal part of the liver was immersed in cold 3.5% glutaraldehyde solution at pH 7.2 for 2hr. After rinsing the tissues several times in cold Soerensen's buffer, each sample was post-fixed in a 1% osmium tetra oxide solution (2hr). The tissues later dehydrated in a series of graded ethanol, placed into propylene oxide and embedded in araldite. Ultrathin

sections were stained with uranyl acetate and lead-citrate. The electron microscope – Zeiss Em 9 S-2 was used for inspecting the hepatocyte ultrastructural changes.

Statistical Analysis: All data are expressed mean values±standard error and subjected to one-way analysis of variants (ANOVA).

RESULTS

Growth Rate: The initial and final weights of flounders observed during the experiment have been recorded in Table 2.

RNA concentrations, Protein Synthesis and RNA/DNA Ratio: The Table 3 shows the synthesis rate in the liver, white muscle and whole body. On the basis of protein synthesis rate the tissues could be arranged in the order: liver > whole body > the white muscles. The liver observed to have twice the rate than the whole body and tenfold greater than the white muscles. The RNA concentration, RNA/DNA ratio and the protein concentration in the liver, white muscles and the whole body are further summarized in Table 3. The protein concentrations have the same pattern as of RNA concentration. The finding showed that this data can be ranked as: whole body> liver> white muscles. The only exception was found in the RNA/DNA ratio where the whole body has value lower than in the liver.

Observations on Hepatic Ultrastructure of Exposed Animals

Normal Cellular Structure: The ultrastructural appearance of hepatocytes of controlled specimen is characterized by the large liver cells with a smooth regular outline and inside dominated by large storage deposits. Numerous glycogen rosettes are distributed uniformly in the liver cells. Most conspicuous is the presence of a prominent centrally located nucleus and equally distinct nucleolus inside it. The nucleus further contains dense clumps of heterochromatin. The organelles are distinct having a visible perinuclear sheath. Layers of rough endoplasmic reticulum (RER) are routinely seen closely associated with mitochondria and peroxisomes (Figures 1 and 2).

Ultrastructural Changes: Many changes are noticed in crude oil exposed specimen and in their targeted tissue, the liver. The hepatocellular cells showed proliferation and stacking (Figure 3) and vesiculation of RER lamellae (Figure 5). There was accumulation of lipid droplets (Figure 3); and a typical mitochondria profiles were seen, there was the presence of unusual membrane formation in the mitochondrial matrix (Figure 4, arrowheads) and further the mitochondria were greatly distorted with matrices becoming coarse and follicular and the cristae appearing swollen and distorted (Figure 4). In normal hepatocytes a conspicuous segmentation of the cytoplasm into a perinuclear organelle rich-zone is seen

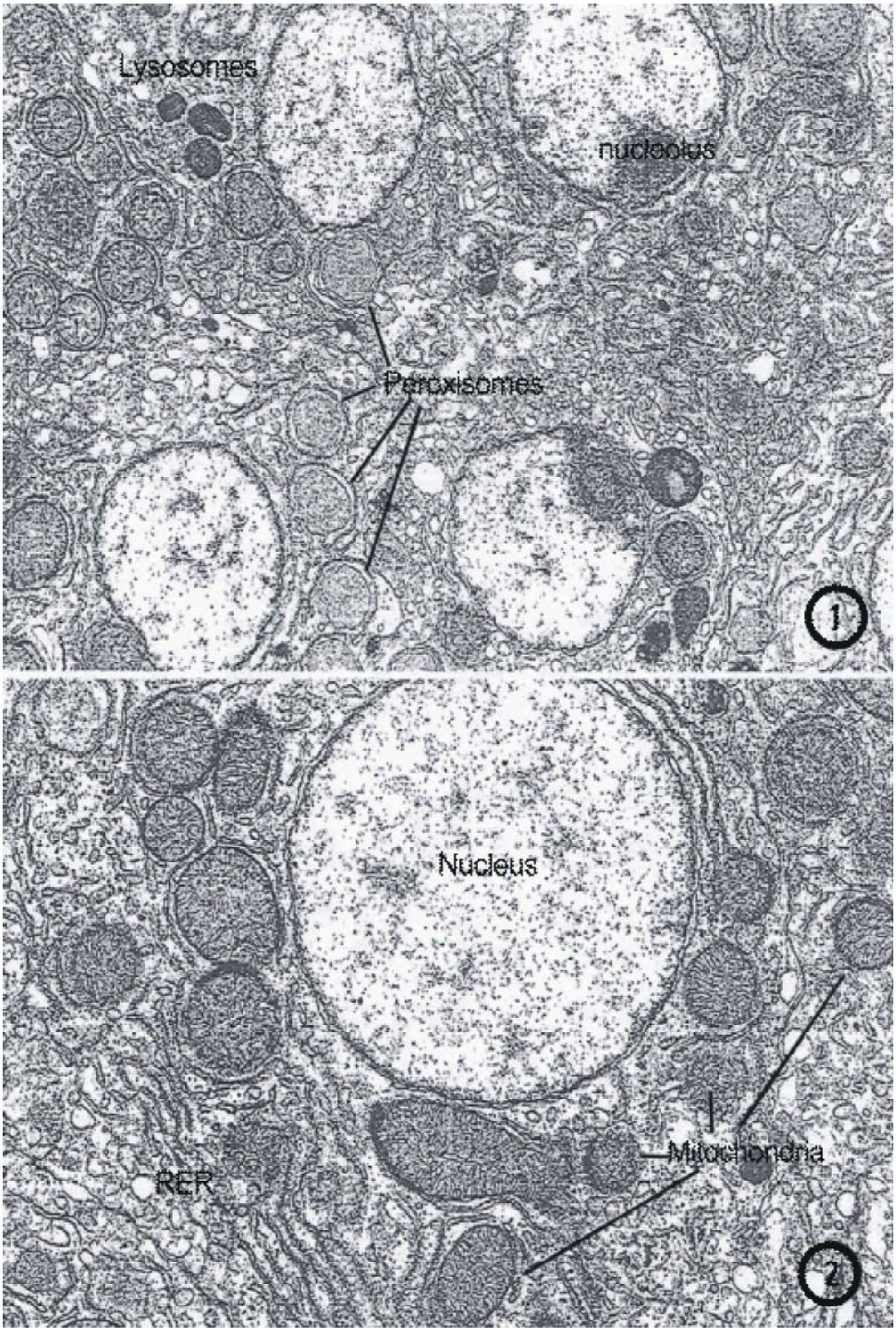
Table 2: Observation on initial and final weights of *Platichthys flesus* during the experimental durations. All fishes were negatively grown.

Specimen	Initial weight W_1(gms)	Final weight W_2 (gms)	*Kg/day	Sex
1	100.9	89	-1.79277	☐
2	195	184	-0.82948	☐
3	214	–	–	☐
4	159	149	-0.92797	☐
5	242	220	-1.36157	☐
6	216	–	–	☐
7	173	–	–	☐
8	215	200	-1.03315	☐
9	163	142	-1.97033	☐
10	166	155	-0.97947	☐
11	132	123	-1.00882	☐
12	142	123	-2.05204	☐

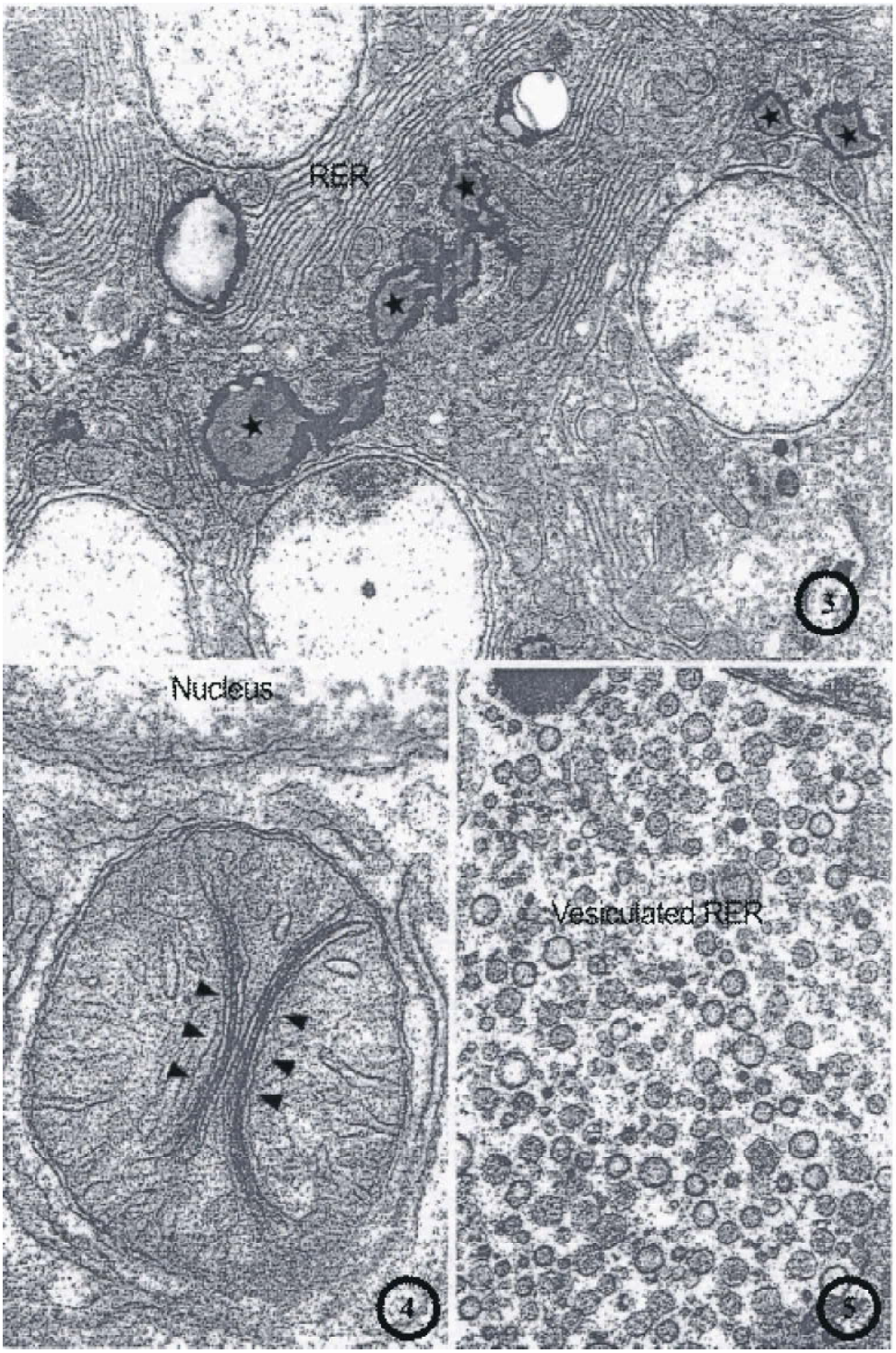
*Growth rate as% per day

Table 3: Protein synthesis and the concentrations of nucleic acids, RNA/DNA ratio in exposed tissues of *Plaichthys flesus*

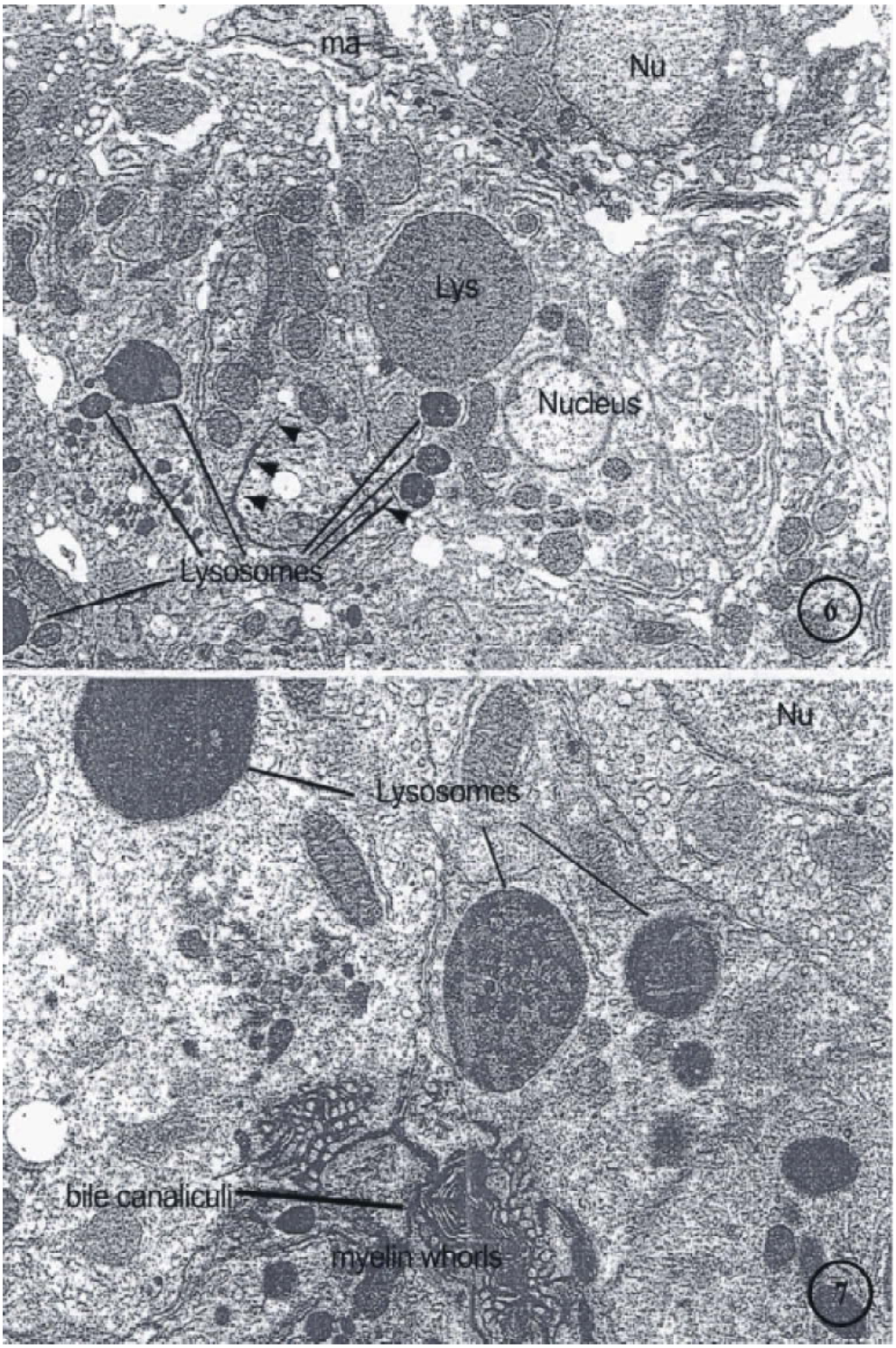
Tissue type	Mean K_s±SE	RNA (μg/g) concentration	Protein (μg/g)	RNA/DNA ratio
Liver	3.10±0.80 (n=11)	857.45±90.632 (n=11)	181.65±5.32 (n=12)	5.017±0.67 (n=11)
White muscles	0.365±0.05 (n=10)	58.02±8.23 (n=10)	124.21±13.04 (n=12)	0.35±0.04 (n=10)
Whole body	1.764±0.26 (n=12)	3480.7±218 (n=12)	326.74±21.81 (n=12)	3.16±0.23 (n=12)



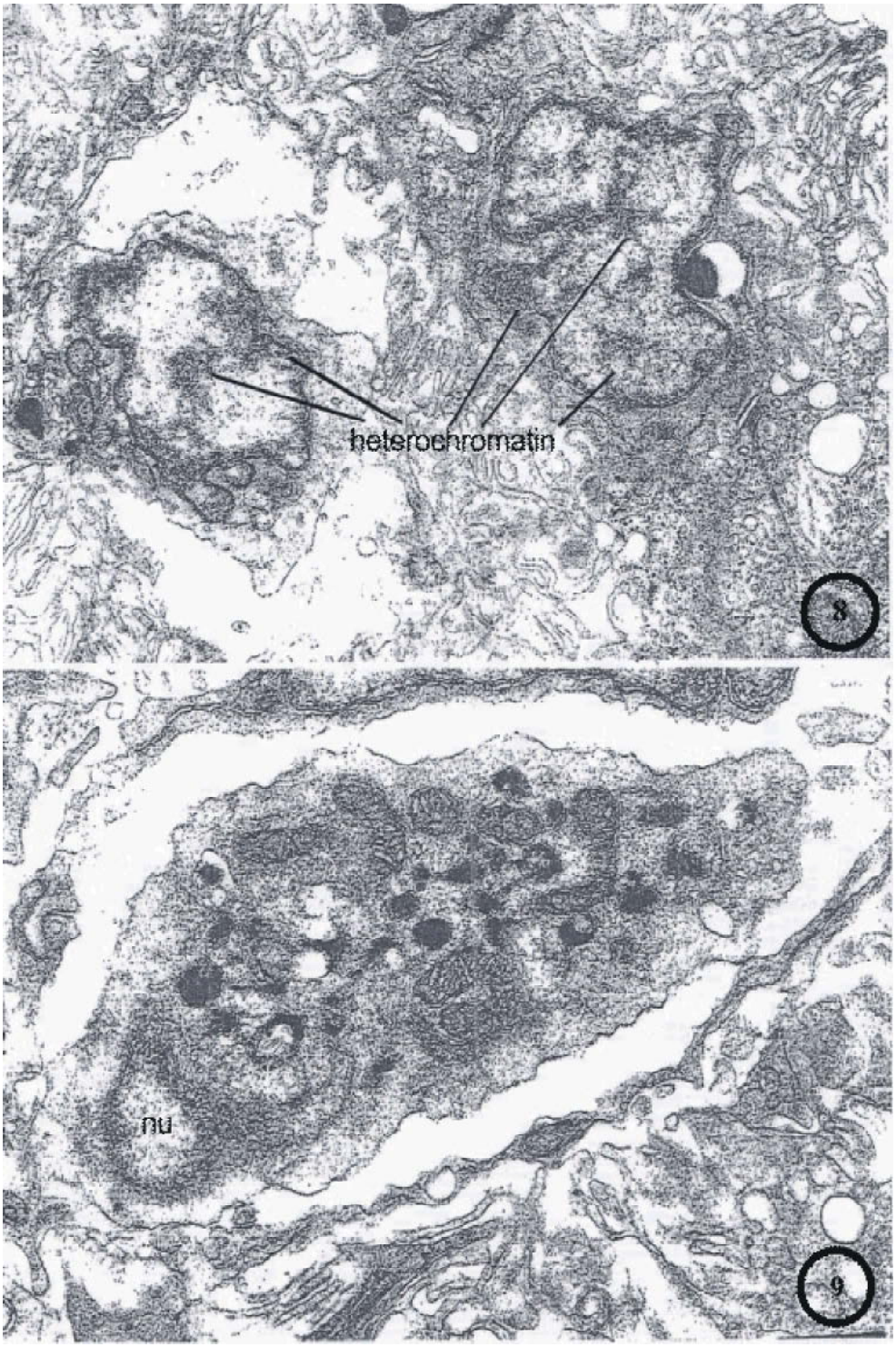
Figs 1 and 2: he cytological appearance of the hepatocytes of control flounder is characterized by the central nucleus with its distinct nucleolus and the organelle-containing perinuclear sheath with a few layers of rough endoplasmic reticulum (RER) closely associated with mitochondria and peroxisomes. Fig. 1: x7,200; Fig. 2: x14,500.



Figs 3-5: Hepatocellular changes induced by crude oil included proliferation and stacking (Fig. 3) as well as vesciculation (Fig. 5) of RER lamellae, accumulation of lipid droplets (Fig. 3, stars), atypical mitochondria profiles and presence of unusual membrane formations in the mitochondrial matrix (Fig. 4, arrowheads). Fig. 3, x7,200; Fig. 4, x54,000; Fig. 5, x24,000.



Figs 6 and 7: In the peribiliary fields of hepatocytes of flounder subjected to crude oil treatment, there was a pronounced proliferation of lysosomes (Lys) in conjunction with an increase in the number and size of Golgi fields (Fig. 6, arrowheads) and presence of myelin whorls in the bile canaliculi (Fig. 7). Nu, nucleus; ma, macrophage. Fig. 6, x5,400; Fig. 7, x17,500.



Figs 8 and 9: The number of macrophages present in the liver parenchyma increased after the crude oil administration in fishes. Macrophages are characterized by an irregularly shaped nucleus (nu) containing appreciable amounts of heterochromatin (Fig. 8) and conspicuous inclusion of hepatocellular organelles (Fig. 9). They invade the liver parenchyma along the biliary tract (Fig. 8) and ingest hepatocytes in the advance stages of necrosis. Fig. 8, x14,500; Fig. 8, x24,000.

typically containing cisternae of rough endoplasmic reticulum (RER)and the mitochondria, peroxisomes and Golgi are filed into the marginal storage areas containing glycogen and lipid deposits.

Certain features were found connected with lysosomes (Lys) in exposed hepatocytes, there was an increase in size in conjunction with an increase in the number and size of Golgi file (Figure 6, arrowheads) and the presence of myeline whorls in the bile canaliculi were conspicuous (Figure 7). Further, the number of macrophages present in the liver or parenchyma found markedly increased in exposed forms. Thus, the macrophages with irregular nucleus, having appreciable amount of heterochromatin could be seen. They invading the liver parenchyma, along the biliary tract seen ingesting hepatocytes as an advanced stage of necrosis (Figure 8). The conspicuous inclusion of hepatocellular organelles is also seen clearly (Figure 9).

The altered organelles was the commonly encountered. The fragmentation of endoplasmic reticulum with some segment showing ill dilated feature was the other result of exposure (Figure 5). The cell membrane of the hepatocytes of the treated fishes wore irregular appearance and often formed a small series of folds. Normal looking nuclei, in the treated forms usually became edematous with heterochromatin densely clumped at the margins (Figure 6). The other crude oil-induced change revealed large lysosomal vacuoles, some containing amyloid bodies and autophagic bodies in the hepatocytes. The autophagic vacuoles and amyloid bodies found generally intact and in numbers (Figure 3); a kind of anomaly rarely seen in the hepatocytes of controlled tissue and were not as large as in exposed forms.

DISCUSSION

The normal functioning of liver is very vital for any organism, the organ is a chemical factory responsible for synthesizing complex molecules. The liver is further involved in breakdown of toxic substances conveyed through hepatic artery. These activities are carried out by the parenchyma cells of the liver and so, become the main causality due to any harmful exposure. The affected mechanism of liver functions seriously suffer when a large number of hepatocytes are damaged. This seems to be the case as of now. It is established that the disturbance of liver functions may further lead to the failure of synthesis of albumin and consequently results in a reduced blood pressure. This situation leads to an accumulation of watery fluid and edema in the peritoneal cavities. Besides, failure of clotting factor synthesis can cause bleeding. The failure of detoxification function may also takes place as the liver fails to convert metabolic waste products and shows the symptoms of hepaticoma. The condition is related to the failure of adequate bile secretion into the elementary tract and the retention of bile to liver, causes jaundice.

The present observations show many kinds of changes in the organelles of the hepatocytes pointing to failure of some of the functions mentioned above. Falling, in the category of distortion, proliferation and loss of architecture, are many organelles. The hepatocytes show organelles, as important as nucleus, mitochondria, ERs with structural changes and most likely the functional disarray of the liver, sometime leading to hepatic cirrhosis. The fish exposure to crude oil may be serious enough when extrapolated with other studies on defective organelles.

There are enough instances confirming this. Short-term exposure of flounders to crude oil do affect the hepatocytes ultrastructures, as in the present case and also seen earlier [6,49]. These authors have reported crude oil linked liver anomalies ranging from gross colour change to subcellular alteration, including effect on proliferation of the endoplasmic reticulum. The increase in the rough endoplasm reticulum observed in these cases could reflect the increase in synthesis of enzymes involved in the detoxification function of liver.

As for the effect of crude oil on growth and synthesis rates of proteins, RNA and DNA – the so called biochemical markers are concerned; a study was taken earlier [4],confirming environment pollution do have an effect on the growth rates and synthesis in the *P. flesus*. These observations are yet again been reinforced by the present work. The crude oil adversely affects both – the growth rates and the synthesis rate of RNA and protein turnover. Dorota *et al.*[30] and Castro *et al.*[50] have reported that the environmental factors; biotic and abiotic can also affect both of these rates; although, the effects of biotic/abiotic factors follows the same pattern in many fishes and thus the effect is usually species-specific. A comprehensive study pointed exactly to the same; the rate of protein turnover in the whole body and white muscles very low compared to liver tissue in fishes [51]. The liver and gill usually show higher values compared to whole body and white muscle and the findings corroborates the observation in the tilapia [4]. The present study also confirmed the individual differences in the tissues.

The present study proposes to extend the observations to other parameters such as enzymatic activities, environmental genotoxicity and cytotoxicity in flounders. These studies have been covered extensively using these methods on different chemicals [31, 52-54]. Furthermore, the direct DNA damage and apoptosis are other parameters of note and so must be included in fishes. As a whole, the importance and feasibility in monitoring systems of *Clarias batarachus* [55] and *Heteropneustes fossilis* [56] has been established in assessing the damage. This would help to arrive at the assessment of damage in a more logistic manner.

CONCLUSION

The flounder, *Platichthys flesus*, was studied to estimate the effect of crude oil on growth and synthesis of RNA and protein and to investigate a plethora of ultrastructural abnormalities in hepatocytes. The results confirmed that hepatocytes were seriously affected by present exposure to crude oil. Many organelles suffer structure deficiencies indicating the interference in the, normal functioning of the liver involving synthesis and detoxification. The organelles disruption and in more serious consequences – the cell rupture was observed. Many lysosomal changes were seen with frequent vacuoles. A large number of secondary lysosomes were also observed denoting active phagocytosis. Mitochondria appear distorted, swollen with disorganized cristae in most cases. In addition, endoplasmic reticulum suffers a loss and even became fragmented. The nucleus – an important controlling point of cell, also undergo changes; looking edematous with heterochromatin appearing dense and clumped. Molecular studies in the present case confirmed that crude oil affected the growth rates, synthesis of RNA and protein turnover significantly. Findings from the study support the active use of nucleic acid as reliable indices of growth. It is further suggested that RNA/DNA ratios have great potential in measuring and assessing the conditions of growth and protein synthesis.

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