Cytotoxicity of Kuwait weathered lake crude oil on rat hepatocytes: a histological and ultrastructural study

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Summary. In the present study, the cytotoxic effects of Kuwaiti weathered crude oil and a potent carcinogen (DMBA) on rat liver cells were examined by light and electron microscopy at each of 4 sampling periods after the start of low dosing (0.5 and 0.2 mg/kg) of feed. Such effects were compared with those of olive oil and uncontaminated food-exposed controls. The results confirm a pronounced cell damage which statistically not significant (p<0.05). In crude oil, the organelle changes were variable and highly comparable to that of DMBA. The nuclei were mostly disintegrated while the cell showed demarcation of cytoplasmic vacuolization, lipid augmentation, and mitochondrial aberrations. The latter showed a remarkable association with the rough endoplasmic reticulum and lipid droplets, and appeared as decayed and diffused structures within the cell matrix. There was no comparable changes in the hepatocytes of animals fed with uncontaminated food except for the formation of lipid droplets in the olive oil-fed groups. Although the animals food was contaminated with Kuwaiti weathered oil formed in 1991 were exposed to extreme seasonal temperatures, yet the residues of such oil led to severe histopathological alterations in the liver cells which were similar to those of DMBA-treated cells. There is the need to pay attention to potential hazardous effects of the crude oil on environments.

Key words: Crude oil, Hepatocyte, Toxicity, Histopathology, DMBA

Introduction

Studies of biological effects including the cytopathological and genotoxical alterations caused by experimental exposures to different toxic substances reveal clear effects on the liver which is one of the prime target tissues among other anatomical organs (Nishizumi, 1970; Hansell and Ecobichon, 1974).

Among petrochemical products, carbon tetrachloride compounds and benzene carcinogenicity and tumorgenicity in rats and human liver had widely reported in many studies (Bong et al., 1985; Maltoni and Scarnato, 1979; Maltoni, 1983; Maltoni et al., 1985, 1987; Gunawardhana et al., 1993; Wilfried et al., 1994). Gasoline, 1, 2butadiene, and kerosene have been shown to cause different malignancies in various organs (Macfarland, 1982; Busey and Cockrell, 1984; Kitchen, 1984; Starck and Vojtech, 1986; Mehlan, 1990, 1992).

Crude oil is potentially, environmentally hazardous as it consists of aliphatic and aromatic hydrocarbons with heterocyclic fractions and impurities such as sulphur and vanadium. The toxic effects of the crude oil are lipid pneumonia, gastrointestinal irritation, and renal tubular nephrosis (Atlas and Bartha, 1973; Hartung and Hunt, 1966; Snyder et al., 1973; Wolfe, 1977). It also induces hepatocellular neoplasms and hepatotoxicity (Macfarland et al., 1984) by alteration of biochemical metabolism (Trump et al., 1984).

Though much information is available on the deleterious effects of crude oil on many species (Albers, 1977; Szaro, 1977; Szaro et al., 1978; Trump et al., 1984; Khan et al., 1987), studies concerning the cytopathological identification are limited. The effects of the oil field damage caused by the Iraqi invasion of the State of Kuwait in 1990 are well known. Over 600 oil wells were set on fire producing hundred of oil lakes. The degree of contaminations of food and water by this massive release of oil remains unresolved. The purpose of this study is to compare pathological effects on rat liver cells after experimental exposure to weathered Kuwaiti crude oil contaminated food with those due to 9,10 dimethylbenz[a]anthracene (DMBA), a known carcinogen causing necrotic lesions, to help evaluate the real and potential dangers facing Kuwait.

Materials and methods

Forty male, 5 week old Sprague-Dawley rats (90-120 g bw) were selected and distributed into 4 groups of 10 animals. They were maintained at 23±2 °C, fed ad libitum on pelleted rodent food (SDS, England). The diet consisted of 20% protein, 5% fat and 53% carbohydrate.
The animals had free access to tap water throughout.

The main control group (I) animals were not exposed to any chemical substance. The second control group (II) animals were exposed to olive oil at the rate of 0.4 g/kg diet. In the first experimental group (III) the rats initially received only pellets contaminated with Kuwaiti crude oil at a dose 0.5 g/kg food. The animals in group (IV) were exposed to 0.2 g/kg diet of 9,10-dimethylbenz[a]anthracene (Sigma) dissolved in 3ml ethanol. Exposure to contaminated food continued for three weeks before returning all animals to normal, uncontaminated food. The experimental design is presented in Table 1.

Groups of four animals were sacrificed 4, 10, 20, 29 and 34 weeks after the start of the experiment. They were anaesthetized with 0.6 ml/100 bw of 25% urethane via intraperitoneal injections. Liver specimens were fixed in Bouin’s fluid, sectioned, stained with H&E and photographed with Olympus AH-3 photomicroscope.

Other liver samples were fixed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.2 for 24 hrs at 0-4 °C for electron microscopy. The tissues were then rinsed in 0.1M sodium cacodylate buffer at pH 7.4 and postfixed in 1% OsO₄ for 1 hr, dehydrated in alcohol and embedded in Araldite M for 48 hrs at 60 °C. Semi-thin sections (1.0 µm) were cut on an LKB ultramicrotome and stained with toluidine blue. Ultrathin sections were double stained with uranyl acetate and lead citrate and examined in a Jeol 1200 EXII electron microscope, operated at 80 kV.

**Statistics**

All the values were expressed as means±SD and involved an independent t-test at the significant level (p<0.05).

**Results**

Table 1. Experimental design of liver treatments.

<table>
<thead>
<tr>
<th>GROUP TREATMENT</th>
<th>bw*/g/gr</th>
<th>bw*/g/gr/WEEK</th>
<th>DOSE/DIET/WEEK</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=10)</td>
<td>4 weeks</td>
<td>10 weeks</td>
<td>20 weeks</td>
</tr>
<tr>
<td>Control</td>
<td>97±8</td>
<td>307±7</td>
<td>437±2</td>
</tr>
<tr>
<td>Olive Oil</td>
<td>107±7</td>
<td>317±14</td>
<td>423±2</td>
</tr>
<tr>
<td>Crude Oil</td>
<td>212±8</td>
<td>293±2</td>
<td>412±24</td>
</tr>
<tr>
<td>DMBA</td>
<td>197±7</td>
<td>369±59</td>
<td>418±14</td>
</tr>
</tbody>
</table>

Values are expressed as means±SD. bw*/g/gr: body weight of animals in grams at the onset of the dosages. bw*/g/gr: body weight of animals in grams per group before sampling. n: number of animals per group. Dose/diet/wk: the given dose in grams per kg rat chow pellets 10 times for 3 respective weeks. No mortality or depression of body weight through the experiment.

Table 2. Morphometric measurements of cell and nuclear sizes in liver sections after treatments.

<table>
<thead>
<tr>
<th>GROUP TREATMENT</th>
<th>CELL SIZE (µm, mean±SD)</th>
<th>NUCLEAR SIZE (µm, mean±SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 weeks</td>
<td>10 weeks</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>19±3</td>
<td>18±3</td>
</tr>
<tr>
<td>Olive Oil</td>
<td>21±3</td>
<td>20±3</td>
</tr>
<tr>
<td>d</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Crude Oil</td>
<td>22±2</td>
<td>19±2</td>
</tr>
<tr>
<td>d</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DMBA</td>
<td>21±3</td>
<td>21±3</td>
</tr>
<tr>
<td>d</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

t: data are expressed as means±SD and evaluated by analysis of variance between and within the groups at significance level 0.05*. n: number of animals per each group. d: number of damaged cells/nuclei.
Fig. 1. Photomicrographs of representative liver stained H&E sections. After 20 weeks:

a. At crude oil treatment the hepatocytes depicting obvious disintegration of the cytoplasm and the nuclei (short arrow). x 800.
b. With DMBA treatment, the hepatocytes are highly vacuolized and disturbed in week 29. x 1,600.
c. The crude oil treatment causes subsequent cytoplasmic vacuolization and damage (arrow) where some cells are seen with rudimentary nuclei (short arrows). x 1,300.
d. With DMBA treatment, the cytoplasmic and nuclei disintegration resemble that in Fig. c. Tissue sampling at 34 weeks. x 1,400.
e. The crude oil treatment indicates further cytoplasmic vacuolization (arrows), where some cells show binucleations (short arrows). x 1,299.
f. With DMBA-treated hepatocytes, an increase of cytoplasmic loss are seen. x 1,299.
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effects of the treatments. In crude oil-treated animals by weeks 4 and 10 after the experiment, the hepatocytes show augmentation of lipid droplets. Many of the droplets were fused together. Others were found associated with the mitochondria forming dense groups. The lysosomes were also observed fused with some inclusions (Fig. 3a). Mitochondria are seen varying in shape and tapered at one end (Fig. 3a inset). In olive oil treatment group, after 4 weeks the cytoplasmic organelles were normal (Fig. 3b), though by week 10 lipid droplets were common (Fig. 3c). In DMBA-treated tissues, the hepatocytes showed remarkable cytoplasmic vacuolization probably in week 4 samples (Fig. 3d) which by week 10, had developed to greater cytoplasmic disintegration.

By week 20 the crude oil-treated cells suggest the presence of diffused mitochondria with clear fusion increasing their heterogeneity. The rough endoplasmic reticulum is densely packed (Fig. 4a) with proliferation of smooth endoplasmic reticulum (Fig. 4a inset). The olive oil-treated cells showed lipid droplets that are highly augmented in the matrix of the hepatocytes (Fig. 4b), while DMBA treatment by week 20 resulted in cytoplasmic disorder leading to severe vacuolization (Fig. 4c).

At week 29 the crude oil treatment showed extensive vacuolization resulting in loss of cytoplasmic contents. Many rough endoplasmic reticulum were seen in close association with mitochondria, some of which appeared in the shape of a dumbell (Fig. 5a). The nuclei commonly had prominent nuclear envelope with unidentified chromatin (Fig. 5b). However, the lipid droplets are highly augmented and densely scattered in the matrix (Fig. 5c). By 34 weeks an advanced stage of

Fig. 2. Pie charts showing the percentage damaged cells and nuclei in weeks 20, 29 and 34 after the commencement of the experiment.

Fig. 3. Electron micrograph showing a series of rat liver cells after 4 and 10 weeks of the experiment. a. At crude oil contaminated food, there is an augmentation and probable fusion of lipid droplets (LD). Some are in a close association with mitochondria (short arrows). Such mitochondria (M) are uniform with an increased matrix density varying in shapes particularly tapered at one end (arrowhead) (inset). Note the nucleus (N) with its nucleolus. Few lysosomes (Ly) appear with some inclusion. B: bile canaliculus. x 10,300. Inset, x 26,500. b. Olive oil treatment after week 4; a portion of liver cells showing normal cytoplasmic matrix with prominent lipid droplets (LD), and smooth endoplasmic reticulum (SER) and part of a nucleus (N). x 13,600. c. With the same treatment at week 10, an area of diffused fine lipid droplets (LD), endoplasmic reticula (SER & RER), and mitochondria (M) are well elaborated. x 15,000. d. With DMBA treatment at week 4, a portion of liver cells show marked vacuolization of cytoplasm. x 6,500. e. At week 10, demarcation of cytoplasmic disintegration is well displayed. The chromatin of the nucleus (N) is clumped. x 6,500.
cell damage was seen where the mitochondria were likely diffused and autolysed (Fig. 5d). The cytoplasm was mostly disturbed (Fig. 5 inset).

DMBA-treated cells showed clear demarcation of areas of the cytoplasmic disintegration by week 29 (Fig. 6a). By week 34 cytoplasmic damage had progressed (Fig. 6b). These two figures might be similar to that of Fig. 4a-d (inset) respectively. Olive oil-treated tissues by week 29 and 34 showed hepatocytes with normal cell components (Fig. 6c). There were no ultrastructural changes observed for the tissue from uncontaminated food control group (Fig. 6d). A summary of these structural alterations is presented in Table 3.

Discussion

This study describes the cytopathological features of the rat liver cells after receiving a 0.5 g crude oil/kg contaminated diet and other of DMBA and olive oil for 3 weeks.

Rats are commonly used as experimental models for monitoring the pathogenesis of human fibrosis, proliferation of hepatic cells induced by some toxicants such as carbon tetrachloride compounds (Herst et al., 1991; Wilfried et al., 1994).

Considerable attention has paid to the description of morphological changes due to hepatotoxicity of crude oil, including the works of Szaro et al. (1977, 1978) on Mallard ducks; Hartung and Hunt (1966) and Snyder et al. (1973) on waterfowl, Khan et al. (1987) on mice. Using 1,2-dichlorobenzene, Gunawardhana (1993) made similar observations on mice.

Light microscopy revealed in the present study that there is noticeable damage to liver cells and their nuclei when exposed to weathered crude oil and DMBA contaminated food. Markedly enlarged cells showing a high nucleus cytoplasm ratio were frequently detected in samples taken at 20, 29, and 34 weeks after the start of the experiments.

Using independent t-test there was no significant difference of the cell and the nuclear sizes of the experimental groups compared to their control (P<0.05).

Table 3. Characterization of cell lesions after treatments.

<table>
<thead>
<tr>
<th>GROUP TREATMENT</th>
<th>TRYP OF LESIONS</th>
<th>SPECIFIC ALTERATION IN CELL ORGANELLES AND INCLUSIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=10)</td>
<td></td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Control Wk</td>
<td>none</td>
<td>normal</td>
</tr>
<tr>
<td>Olive Oil Wk</td>
<td>lipid augmentation</td>
<td>normal</td>
</tr>
<tr>
<td>Crude Oil Wk</td>
<td>Vacuolization</td>
<td>abnormal</td>
</tr>
<tr>
<td>DMBA Wk</td>
<td>vacu./disin.</td>
<td>abnormal</td>
</tr>
</tbody>
</table>

Wk: week after the commencement of the experiment. Abnormal mitochondria: the organelles were either dumbbell-shaped or fused with each other with unidentified or damaged cristae. Abnormal nuclei: the organelles with disturbed chromatin. Abnormal nucleoli: the organelles were accentric or completely disappeared. Some nuclei were found with double nucleoli. Abnormal SER: the organelles were highly vesiculated and proliferated. Abnormal RER: the organelles were fragmented and aggregated. Abnormal lysosomes: the organelles were impacted to other inclusions. Abnormal lipids: lipid droplets were highly augmented and probably fused. /: augmentation in crude oil treatment week sampling. Vacu./disin.: vacuolization / disintegration.

Fig. 4. Electron micrographs of liver cells after 20 weeks of the commencement of the experiment. a. Hepatocyte portion after crude oil food contamination showing accumulation of lipid droplets (LD) devoid of a limiting membrane. The mitochondria (M) are diffused showing remarkable fusion (arrows) with an increase in their heterogenetical shapes. Dense granulated stacks of endoplasmic reticulum (RER) are present. Again the cytoplasm depicts presence of SER (inset). The nuclei (N) are normal. x 24,000. Inset, x 33,000. b. In olive oil treatment, the lipid droplets (LD) are highly augmented. They vary in size and many are evidently fused. x 4,200. c. In DMBA treatment, a portion of two liver cells showing late-cytoplasmic disorganization in the left cell (LC) and a prominent cytoplasmic vacuolization (RC). Numerous lipid droplets (LD) are present. Meanwhile chromatin are densely seen in the vicinity of the nuclear envelope. x 8,000.

Fig. 5. Electron micrographs of liver cells processed at 29 and 34 weeks after crude oil food poisoning. a. Three hepatic cells at week 29, loss of cytoplasmic contents in the upper cell (UC), and partially so in the right cell (RC) which has a peripherally cut nucleus (N). The cisternae of the endoplasmic reticulum are arranged into parallel arrays (RER), many of which are closely associated or even encircle the mitochondria as shown in the left cell (LC) (short arrows). Same feature is encountered in UC and RC, but more extensively. Some mitochondria exhibit dumbbell shape (arrowhead). x 7,100. b. The nuclear envelope is prominent due to the disruption of the chromatin. x 7,500. c. Part of the cell showing remarkable augmentation of lipid droplets (LD). x 11,000. d. At week 34, a portion of hepatic cell showing an advanced stage of damage. The cisternae of the rough endoplasmic reticulum (RER) clearly surround the mitochondria (M) (arrowheads). Some of them are highly diffused with unseen cisternae and cytoplasmic matrix (Short arrow). Again, other cells depict much damaged cytoplasmic proliferation, where the nucleus (N) have a prominent nuclear envelope (inset). x 4,300. Inset, x 13,500.
The results further reveal that the percentage of the damaged cells and nuclei were high in crude oil and DMBA-treated tissue (35-50%) at all sampling periods, while the olive oil and non-contaminated control tissue showed little change at 20, 29, and 34 weeks respectively.

One important finding was the presence of distinctly enlarged cells as evidenced in the H&E-stained liver sections, possessing 2 or 3 eccentrically located nuclei. Although this has been reported in normal hepatocytes with binucleation and polyploidy which increased with age (Styles et al., 1987), the present study addresses the disintegration of the genetic materials as well as the nuclei which may be considered as being in a pathological condition after exposure to weathered crude oil. In case of DMBA-treated groups, the nuclear components were similarly disturbed.

Ultrastructural analysis of the weathered crude oil samples, showed characteristic features of liver damage such as extensive vacuolization, degranulation, disintegration and loss of cytoplasm. This result agrees with the previous studies on crude oil (Szaro et al., 1978), 1.2-dichlorobenzene (Gunawardhana, 1993), polychloronated biphenyl congeners (Macelllan et al., 1994a-c), R-33 benzene fraction (Bong et al., 1985), Prudhoe Bay crude oil (Khan et al., 1987), carbon tetrachloride (Irita et al., 1984), trichloroethylene (Heining and Hoffman, 1993), benzene and gasoline (Mehlman, 1990, 1992). In the present study weathered crude oil was compared with DMBA as the latter was considered a dependable indicator of cell and nuclear damage due to its carcinogenicity (Fischer et al., 1991; Sadek and Hayat, 1996).

The results underline the considerable lipid augmentation which were evident in the crude oil treated samples. Also the presence of condensed, sometimes fused mitochondria with different shapes and sizes having broken and disturbed cristae were often common in destroyed matrix. The RER was highly stacked in parallel rows with dilated cristae as reported in other studies (Nishizumi, 1970; Hansell and Ecobicon, 1974; Lin et al., 1979; Macelllan et al., 1994a-c; Wilfried et al., 1994).

The ultrastructural aberrations may have affected the mitochondrial energy production efficiency reducing oxidative metabolism resulting in an accumulation of lipid droplets of the hepatocytes especially in the crude oil samples. A similar situation has been reported for cytopathological condition (Khan et al., 1986; Schecter et al., 1984; Cullen et al., 1996). This accumulation of lipid may have significantly disturbed the cellular lipid metabolism (Kyle and Farber, 1991; Cullen et al., 1996).

The lipophilic cell membranes are such that the toxicant may act on specific sites as reproted in tissues exposed to crude oil by Leighton et al. (1985), to orthophenyl phenol by Nakagawa et al. (1992) and to polychlorinated penyls by Macelllan (1994a,b).

The free radicals of carbon tetrachloride has shown to induce proliferation of endoplasmic reticulum, lipid peroxidation, inhibition of protein synthesis and mitochondrial diffusion which may initiate cell lesions (Reynolds, 1972; Recknagel et al., 1982; Comporti, 1985; Brent et al., 1993).

The features of the liver cytopathogenesis induced by weathered crude oil and DMBA are shown in Table 3. In comparison, the control and olive oil groups showed normal anatomical features; viz: spherical nuclei with prominent nucleoli, round or elongated mitochondria, and normally arranged endoplasmic reticula (Sadek and Hayat, 1996). The olive oil samples were characterized by having more lipid droplets with augmentation sites.

In conclusion, the present study shows the histological effects of Kuwaiti weathered crude oil on the hepatocytes of the rat. It is important to realize that the crude oil was collected from an oil lake in 1994 where it had been exposed to extreme temperatures which rise to 50 °C in the shade for the months of June to August. The oil lake had been formed due to the rupture of an oil pipeline after being blown up by the Iraqi forces in 1991. This exposure to sunlight will have removed most of the volatile elements of oil which have often been regarded as some of the most harmful to Man and other species. Thus the histological changes reported must be due to other factors in the oil such as the high sulphur levels, the presence of heavy metals such as vanadium and/or toxic factors in the tar element of the crude oil. The significance of these findings is that after many years of exposure to sun and seasonal rainfall which has exceeded 200 mm in recent years, the crude oil in these man-made lakes remains a serious health hazard.

The effects of low levels of contaminated of food on the liver reveals marked vacuolization of cells, nuclear disintegration and general cellular damage. The histopathological changes seen in the crude oil-fed animals were similar to those seen in animals fed on the known carcinogen DMBA. Thus it is vital that further studies investigate both the specific mechanism of the damage, the specific agents causing that damage and the probability of each agent being bioaccumulated through grasses and the grazing food chain.

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