Journal of Anatolian Environmental and Animal Sciences
(*Anadolu Çevre ve Hayvancılık Bilimleri Dergisi*)
DOI: https://doi.org/10.35229/jaes.1294687

JAES
Year: 8, No: 3, 2023 (283-290)
AÇEH
Yıl: 8, Sayı: 3, 2023 (283-290)

ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Histopathological Evaluation Of Liver Changes In Rainbow Trout After Diethlynitrosamine (DEN) Exposure

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Geliş/Received: *10.05.2023* ***Kabul/Accepted:*** *04.08.2023* ***Yayın/Puplished:*** *30.09.2023*

How to cite: **Yardimci, B., Onuk, E.E. & Pekmezci, G.Z. (2023).** Histopathological evaluation of liver changes in rainbow trout after diethlynitrosamine (DEN) exposure. *J. Anatolian Env. and Anim. Sciences*, ***8***(3), 283-290. https://doi.org/10.35229/jaes.1294687
Atıf yapmak için: **Yardimci, B., Onuk, E.E. & Pekmezci, G.Z. (2023).** Dietilnitrozamin (DEN) uygulanan gökkuşağı alabalıklarında karaciğer değişikliklerinin histopatolojik değerlendirilmesi. *Anadolu Çev. ve Hay. Dergisi*, ***8***(3), 283-290. https://doi.org/10.35229/jaes.1294687

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Abstract: The aim of this study was to determine the hepatocarcinogenic effect of diethylnitrosamine in rainbow trout (*Oncorhynchus mykiss*), the most cultivated and consumed freshwater fish. In this study, 120 fish weighing 50-70 g each were used. Randomly selected fish were divided into 3 dosage groups and a control group that contained 10 fish per group. Groups were named as A group on the 15th day, group B on the 30th day and group C on the 45th day. In addition, since 3 different doses were administered to each group, they were divided into A1, A2, A3, B1, B2, B3, C1, C2, C3. Diethylnitrosaminewas dissolved in 0.1 ml of dimethylsulfoxideat 50 mg/kg, 100 mg/kg and 150 mg/kg, and respectively administered to each of the dosage groups intraperitoneally. On the 15th, 30th and 45th days of the study, 10 fish from each study group were euthanised while under sedation, and necropsies were performed. For histopathological and immunohistochemical examinations, tissues were routinely processed, and sections were stained with haematoxylin-eosin and examined by light microscopy. Histology revealed hyperaemia, parenchymal degeneration, mononuclear cell infusion, necrosis, bile ducts hyperplasia, cholangiofibrosis and fatty degeneration of the liver. In immunohistochemical staining, CYP1A was slightly positive in the bile duct epithelium in the C2, A3, B3 and C3 groups, and negative in the other groups. Cytokeratin 18 showed negative staining in all groups. Mild positive staining in the bile duct epithelium, A3, B3 and C3 and negative staining in other groups for glutathione-S-transferase. It was concluded that diethylnitrosamine causes significant damage to liver tissue in fish, even in short-term applications, and may cause neoplastic changes in liver cells over the long term.

Keywords: Diethylnitrosamine, hepatocarcinogen, histopathology, immunohistochemistry, rainbow trout (*Oncorhynchus mykiss*).

Dietilnitrozamin (DEN) Uygulanan Gökkuşağı Alabalıklarında Karaciğer Değişikliklerinin Histopatolojik Değerlendirilmesi

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Öz: Bu çalışmanın amacı, endüstriyel ve zirai faaliyetlerde kullanılan birçok ürünün içerisinde ve atıklarında bulunan, canlılarda karsinojenik özellikteki, dietilnitrozamin (DEN) in tatlı sularda en çok yetiştiriciliği yapılan ve tüketilen balık olan gökkuşağı alabalığındaki hepatokarsinojen etkisini ortaya koymaktı. Çalışmada, 50-70 gr ağırlığındaki 120 adet balık kullanıldı. Rastgele seçilen balıklar her grupta 10 balık olacak şekilde ve her doz için 3'erli gruplara ayrıldı. Gruplar 15. gün A, 30. gün B ve 45. gün ise C grubu olarak adlandırıldı. Ayrıca her gruba 3 farklı doz uygulaması yapıldığı için A1, A2, A3, B1, B2, B3, C1, C2, C3 olarak ayrıldı. 50 mg/kg, 100 mg/kg ve 150 mg/kg olacak şekilde DEN (dietilnitrozamin) 0,1 ml DMSO (dimetilsülfoksit) da çözdürülerek intraperitoneal (ip) yolla verildi. 15, 30 ve 45. günlerde, her bir grupta bulunan balıklara sedasyon uygulanarak ötenazileri gerçekleştirilip nekropsileri yapıldı. Histopatolojik ve immunohistokimyasal inceleme için örnekler alınarak rutin doku takibi prosedürü uygulandı, ışık mikroskobunda incelendi. Histolojik olarak, karaciğerde hiperemi, parankimal dejenerasyon, mononükleer hücre infiltrasyonu, nekroz, safra kanallarında hiperplazi, kolangiofibrozis ve yağlı dejenerasyon bulguları gözlendi. İmmunohistokimyasal boyamada, CYP1A yönünden C2, A3, B3 ve C3 gruplarında safra kanalı epitellerinde hafif pozitif, diğer gruplarda ise negatif boyanma; Cytokeratin 18 yönünden tüm gruplar negatif boyanma ve GST yönünden A3, B3 ve C3 gruplarında safra kanalı epitellerinde hafif pozitif, gruplarda ise negatif boyanma tespit edildi.Sonuç olarak, dietilnitrozaminin balıklarda kısa süreli uygulamalarda bile karaciğer dokusunda belirgin hasara neden olduğu ve uzun dönemde karaciğer hücrelerinde neoplastik değişikliklere neden olabileceği kanısına varıldı.

Anahtar kelimeler: Dietilnitrozamin, gökkuşağı alabalığı (*Oncorhynchus mykiss*), hepatokarsinojen, histopatoloji, immunohistokimya.

INTRODUCTION

Water is essential for life for all living things. Everyone needs clean and healthy water. Water is polluted for natural and artificial reasons, this results in the emergence of more than one water pollutant. The discharge of heavy metals, along with chemicals, which are the most important anthropogenic pollutants, through industries and factories are the main sources of water pollution. (Singh et al., 2019; Manalo et al., 2023) Fish have attracted attention as valuable models to be used as an early warning system for the detection of carcinogens in aquatic environments over the past 30 years. Studies carried out in a laboratory setting also support the connection between cancer and the growth of various water pollutants in fish (Couch et al., 1985; Dunn et al., 1987; Varanasi et al., 1987; Gardner, 1988; Metcalfe et al., 1988; Varanasi et al., 1989; Maccubbin et al., 1990; Black et al.,1991). Many tumor suppressor genes found in humans have also been expressed in fish. Various tumor types, such as hepatocellular adenomas and carcinomas, are affected by dysregulation of the expression of these genes, which act as regulators of cell cycle, proliferation, and malignant changes. Studies have shown that these gene structures are similar between fish and humans, and it has been concluded that fish can be a model for studies on humans (Chen et al., 2014; Dias Guerreiro, 2019; Etchin et al., 2011; Howe et al., 2013). Moreover, non-mammalian vertebrate models, various fish species have been examined as indicators of ecological contamination in order to understand the mechanisms of development and prevention of cancers in humans (Bailey et al., 1996).

At the basis of cancer development, mutations that biologically stimulate events, such as cell proliferation, proliferation control and differentiation, must all come together. In this process, the cells undergo many changes, and as a result, tumour cells can rapidly and unlimitedly spread to the surrounding tissues. There are also oncogenic mutations targeting intercellular signal transduction pathways and proteins in carcinogenesis. This makes the cell cycle control points inactive and plays an active role in tumour formation, invasion and metastasis (Dogan et al., 2004; Ustuner, 2006).

Hepatocellular carcinomas are among the most common tumors in the world and it is estimated that there has been a significant increase especially in the last decade, with 500,000 to 1,000,000 new cases of HCC reported each year, and their causes include Aflatoxin B1 and Hepatitis B-C viruses, and chemicals such as diethylnitrosamine (DEN), phenobarbital, 2-acetylaminofluorene (2-AAF) and alcohol (Dias Guerreiro, 2019; Kuroda et al., 2002; Thirunavukkarasu et al., 2003; Ustuner, 2006; Montella et al., 2015). The hepatocarcinogenicity of DEN was experimentally demonstrated for the first time by Stanton (1965) in zebrafish (*Danio rerio*) (Stanton, 1965; Bailey et al., 1996). In the studies carried out, a mutation was detected in the 61st codon of the Ha-Ras proto-oncogene, which served in the pathway of DEN's mitogen-activated protein kinases (MAPK). This mutation has also been found to induce tumour formation in the liver (Frey et al., 2000; Aydinlik et al., 2001; Ustuner, 2006).

DEN is a carcinogenic substance that is formed from insecticides and nitrates used in agriculture; can be found in cigarette smoke, alcoholic beverages and processed meat products; and can also occur during the metabolism of certain drugs in the liver, as well as in the reaction of nitrates in foods with secondary and tertiary amines in the stomach (Chiarello et al., 1998). DEN is one of the nitrosamine compounds that cause hepatocellular carcinoma. According to doses of DEN, rising of 8-OH 2 'deoxyguanosine (8-OHdG) levels indicate oxidative DNA damage in hepatocytes. It was determined that the 8-OHdG level increased in the early period (6 hours after DEN administration). In tumour formation, there is a relationship between lipid peroxidation and environmental free radicals. In addition, antioxidant protective effects are used against hepatic carcinomas formed due to DEN. Free radicals originating from DEN are formed microsomal metabolism during in vitro trials, however, in vivo studies, the relationship of elevation of free radicals with tissue damage has not been fully proven yet. Based on this, DEN's cytochrome P450 reductase pathway was observed to be metabolized and, as a result, free radicals were formed (Yamada et al., 2006). DEN is metabolised by the enzymes of the monooxygenase system bound to the cytochrome P450reductase enzyme, but reactive intermediates, which have little relevance to the catalytic sites of the binding enzymes, cause necrosis, mutation and cancer by forming covalent bonds with important cell components (Daoust et al., 1986). It also states that lipid-bound free radicals in the liver occur within 1-24 hours following the administration of DEN (Yamada et al., 2006).

In experimental studies, chemicals with carcinogenic effects were added to fish feed, given to embryos and offspring in the form of baths or microinjections, or administered intraperitoneally or through a gavage. Carcinogenic effects were found following all application methods (Bailey et al., 1996). Presently, in studies performed with DEN, the microinjection method is preferred for embryos and juveniles, and bath and food administration are preferred for adults (Hendricks et al., 1984; Lee et al., 1989; Bailey et al., 1996; Bunton, 1996).

In the examinations performed, non-neoplastic lesions, such as fatty degeneration of the liver, basophilic and eosinophilic cell hyperplasia, bile duct epithelium proliferation, cholangiofibrosis and pancreatic metaplasia were observed. Furthermore, neoplastic lesions, such as hepatocellular carcinoma, hepatocellular adenoma, cholangiocarcinoma, cholangioma, hemangiopericytoma have been identified (Hendricks et al., 1984; Lee et al., 1989; Hendricks et al., 1994; Bunton, 1996).

In this study, DEN, which is a carcinogen found in many products used in industrial and agricultural activities and in their waste, was used to reveal the hepatocarcinogenic effect in rainbow trout (*Oncorhynchus mykiss*), which is the most cultivated and consumed freshwater fish. Rainbow trout was used as a model for DEN toxicity in the present study.

MATERIAL AND METHOD

The 120 rainbow trout used in this study were obtained from a commercial trout farm, and weighed 50-70 g each (The animal experiments in this study were performed in accordance with relevant national and international guidelines. All the experiments were approved by the Ethics Committee of Ondokuz Mayis University (2012/71)). In the experiment, 12 300-liter polyester tanks were used. These tanks were disinfected before the study and filled with tap water, which was rested for at least 48 hours prior to the beginning of study and was free of chlorine.

Experimental protocol: Fish were placed in stock tanks in the laboratory for adaptation purposes 15 days before the start of the study. Later, the randomly selected fish were transferred to 12 300-liter polyester tanks, in which the 10 fish took place. The randomly selected fish were divided into 3 dosage groups and 1 control group containing 30 fish each. Groups were named as A group on the 15th day, group B on the 30th day and group C on the 45th day. In addition, since 3 different doses were administered to each group, they were divided into A1, A2, A3, B1, B2, B3, C1, C2, C3. Mild sedation was administered intraperitoneally (ip) to each dosage group by dissolving DEN in 0.1 ml dimethylsulfoxide (DMSO) at 50 mg/kg, 100 mg/kg and 150 mg/kg, respectively. In the control group, only 0.1 ml of DMSO was injected in the same way. Each trial (10 fish) was done in separate tanks. During the trial period, the fish were fed with trout growing feed at a rate of 2% of their body weight per day (Table 1). During the experiment, the pH of the water was between 7.4 mg/L and 7.7 mg/L, and the dissolved oxygen concentration of the water (determined weekly) was between 8.2 mg/L and 9.4 mg/L.

Table 1. Groups and diethylnitrosamine (DEN) doses used in the experiment.

	Group name and number of fish			Total number of fish
	15th day	30th day	45th day	
Control (dimethylsulfoxide ,DMSO)	10	10	10	30
Group 1 (A) (50 mg/kg, DEN)	A1-10	A2-10	A3-10	30
Group 2 (B) (100 mg/kg, DEN)	B1-10	B2-10	B3-10	30
Group 3 (C) (150 mg/kg, DEN)	C1-10	C2-10	C3-10	30

Macroscopical and histopathological examination: Following the administration of DEN, 10 fish from each study group were euthanised while under sedation by MS222 (30mg/lt) on the 15th, 30th and 45th days of the study, and necropsies were conducted. Following the necropsy, tissue samples from the skin, gills, muscles, liver, kidneys, spleen, hearth, stomach, intestines, brain, eyes and gonads were taken for histopathological examination and fixed in 10% neutral formaldehyde solution. For this study, only the liver samples taken were routinely processed and embedded in paraffin. Tissue sections 4-6 µ thick in width were stained with haematoxyline-eosin and examined using light microscopy. After the evaluation, the prepared tissues that were deemed suitable were immunohistochemically stained for CYP1A1, Cytokeratin 18 and glutathione-S-transferase-p (GST-p) antibodies and evaluated under the light microscope (Eclipse E600, Nikon).

Immunohistochemistry: In this study, the streptavidin-biotin peroxidase method was used for immunohistochemical method. Sections that were 4µ thick were taken from paraffin blocks and placed on slides coated with 3-Aminopropyl triethoxysilane (APES). Deparaffinization and dehydration were performed for each section. Then, all the sections were kept in a 0.3% solution of hydrogen peroxide in methanol for 30 minutes in order to prevent endogenous peroxide activity. Microwave or pronase or trypsin were used to elicit antigenic receptors where necessary. After washing the sections with phosphate buffer solution (PBS), the sections were incubated with different rates of diluted primary antibodies in PBS at different times and temperatures. After washing with PBS again, sections were incubated with monoclonal mouse cytokeratin 18 (Sigma-Aldrich, 100 µl) polyclonal rabbit GST (Sigma-Aldrich, 25 µl) and polyclonal rabbit cytochrome P450 reductase, family 1, subfamily A (CYP1A) (Sigma-Aldrich, 100 µl) for 30 minutes with biotinylated goat anti-rabbit immunoglobulin (1:300) at room temperature. Subsequently, all sections were washed with PBS and incubated with streptavidin-peroxidase complex (Dako; 1:300) for 30 minutes. Labelling was ‘visualized’ with 3-amino-9-ethylcarbazole (AEC; Golden Bridge Int., Life Science) or 3.3'-diaminobenzidine tetrahydrochloride (DAB) prepared in 0.035% of PBS as the chromogen. Sections were stained with Gill’s Hematoxylen for 20 seconds and washed in tap water. Later, the sections were covered with a water-based

or normal immunmount and evaluated under a light microscope.

The sample size of this research was decided by performing power analysis in the G'Power 3.1 statistical program. The error probable (α) was taken as 0.05 and the statistical power (1-β) as 0.80.

RESULTS

Clinical and macroscopical findings: In the 15-day trial, on the 1st and 8th day of the experiment, 1 fish from the B1 group died, and on the 12th and 14th day of the experiment1 fish from the C1 group died. Necropsies were performed immediately on the fish that died during the trial, and others were euthanized according to protocol on the last day of the trial. In the B1 group, the necropsy of the fish that died on the 1st day of the experiment showed only mild petechial haemorrhages at the injection site and a yellowish, gelatinous injection material. In the fish that died on the 8th day, mild tail fin rot, ascites and anal prolapse were observed. In addition, 1 of the fish had a rupture on its abdominal wall. In the C1 group, the fish that died on the 12th day of the experiment exhibited haemorrhages in the pelvic and pectoral fin bases, injection zone, and adipose tissue in the abdominal cavity. Fish that died on the 14th day exhibited lightening of the skin colour, mild tail fin rot, mucus accumulation between the gill lamellae and slight haemorrhages at the injection site.

At the end of the experiment, in the A1 group, darkening of the skin colour, in the abdominal cavity the colour of the liver was slightly pale, with marked haemorrhaging at the injection site and adipose tissue was observed. In the B1 group, darkening of the skin colour, haemorrhaging in the pectoral and pelvic fin bases in 1 fish, and haemorrhages at the injection site and surrounding tissues in the abdominal cavity were observed. The liver was pale and had haemorrhagic foci. In the C1 group, lightening of the skin colour and tail fin rot was observed. In addition, 1 fish showed a slightly erosive lesion, approximately 1 cm in diameter, on itsback skin. Haemorrhages was observed at the injection site and surrounding adipose tissue, and the pyloric caeca and serosa in the abdominal cavity in all fish of Group C1.

In the 30-day trial, on the 12th day of the experiment, 1 fish from the A2 group died, and on the 16th day, 1 fish from the A2 group and 1 fish from the B2 group died. The necropsies found that there was mild acites in all 3 fish and petechial haemorrhages at the injection site was observed. At the end of the experiment, in fish in the A2 group, darkening of the skin colour and paleness in liver colour were observed. In the B2 group, darkening of the skin colour, mild tail fin rot and paleness in liver colour were observed. In the C2 group, darkening of the skin colour, foci of skin erosions and grey-white areas in the liver were encountered.

In the 45-day trial, on the 4th day of the experiment, 1 fish died in the C3 group. The necropsy showed only mild petechial haemorrhages and a yellowish, gelatinous material was found at the injection site. At the end of the experiment, darkening of the skin colour was observed in all groups, more prominent in B3 and C3 groups, was haemorrhages in the liver, as well as pale areas on the margins of the liver.

***Histopathological findings*:** In the 15-day trial, mild hyperaemia in 3 fish in the A1 group; moderate hyperaemia in 1 fish in the A1 group and 2 fish in the C1 group; and severe hyperaemia in 1 fish in the B1 group and 2 fish in the C1 group was observed. In the hepatocytes, parenchymal and fatty degeneration were as follows: mild in 2 fish from the B1 group, and moderate in 4 and severe in 1 from the C1 group. Mild mononuclear cell infiltration was observed around the bile ducts in only 1 fish in the B1 group. Hyperplasia was noted in the bile duct epithelium in 2 fish - 1 from the B1 group and 1 from the C1 group (Figure 1a).

In the 30-day trial, mild hyperaemia in 1 fish in the C2 group; moderate hyperaemia in 3 fish in the A2 group, 3 fish in the B2 group and 2 fish in the C2 group; and severe hyperaemia was observed in 3 fish in the C2 group. In the hepatocytes, parenchymal and fatty degeneration were as follows: mild in 1 fish from the B2 group; moderate in 2 fish from the A1 group, 3 fish from the B2 group and 2 fish from the C2 group; and severe in 3 fish from the C1 group. Focal necrosis was observed in the hepatocytes of 2 fish in the A2 group and 1 fish in the C2 group. In addition, hyperplasia in the bile duct epithelium in 1 fish in the B2 group and 2 fish in the C2 group, and necrosis of melanomacrophages in 1 fish in the C2 group were observed (Figure 1b-d).

In the 45-day trial, mild hyperaemia was observed in 3 fish in the A3 group and 4 fish in the B3 group. In the hepatocytes, parenchymal and fatty degeneration were as follows: moderate in 7 fish in the A3 group, and severe in 8 fish in the B3 group and 10 fish in the C3 group. Focal mononuclear cell infiltration was observed around the bile ducts in 2 fish in the A3 group and 1 fish in the B3 group. In the hepatocytes, necrosis was observed as follows: moderate in 7 fish in the A3 group, and severe in 8 fish in the B3 group and 10 fish in the C3 group. Hyperplasia in the bile duct epithelium was noted in 3 fish in the A3 group, 2 fish in the B3 group and 5 fish in the C3 group. In addition, cholangiofibrosis in 1 fish in the A3 group, 2 fish in the B3 group and 2 fish in the C3 group, and necrosis of melanomacrophages in 2 fish in the A3 group and 1 fish in the C3 group were observed (Figure 1e, f).

Immunohistochemical findings: In the examination performed as a result of immunohistochemical staining, in the C2, A3, B3 and C3 groups in terms of CYP1A, a mild positive staining was seen in the bile duct epithelium, whereas the other groups stained negative (Figure 2a-d). All groups exhibited negative staining in terms of cytokeratin 18, and a mild positive staining was seen in the bile duct epithelium in the A3, B3 and C3 groups, whereas the other groups stained negative in terms of GST (Figure 3a, b).

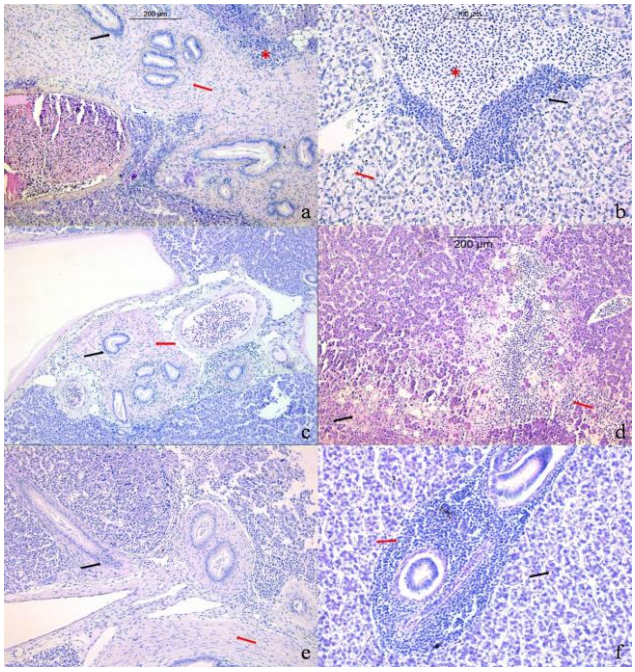

Figure 1. (a) GroupC1, hyperplasia in the bile duct epithelium (black arrow) fibrosis (red arrow) and mononuclear cell infiltration (asterisk) x100, HXE. **(b)** Group A2, severe hyperaemia (asterisk), mononuclear cell infiltration around the vein (black arrow) and vacuolar degeneration in hepatocytes (red arrow) x200, HXE. **(c)** Group B2, hyperplasia in the bile duct epithelium (black arrow), fibrosis (red arrow) x100, HXE. **(d)** Group C2, necrosis in hepatocytes (black arrow) and and mononuclear cell infiltration (red arrow) x100, HXE. **(e)** Group A3, hyperplasia in the bile duct epithelium (black arrow) fibrosis (red arrow) x100, HXE. **(f)** Group B3, mononuclear cel linfiltration around the bile ducts (red arrow) and parenchymal degenaration of hepatocytes (black arrow) x200, HXE.

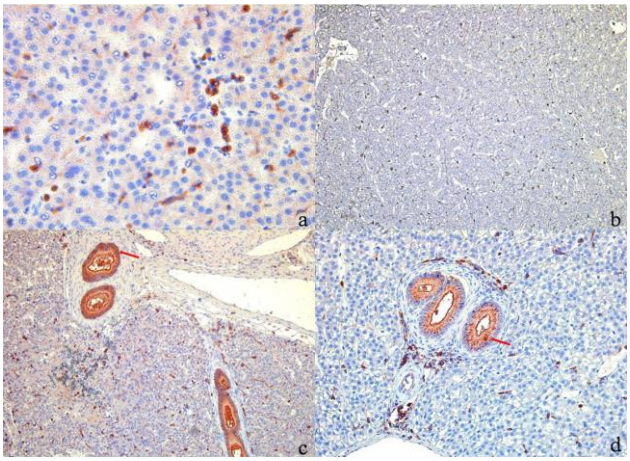

Figure. 2. CYP1A; **(a)** Control group x400. **(b)** Group B1 negative x100. **(c)** Group A3 mild positive in bile duct epithelium (red arrow) x100. **(d)** GroupC3 mild positive in bile duct epithelium (red arrow) x200, ABC method.

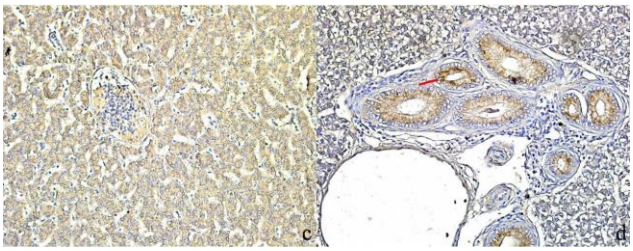

Figure 3. GST; **(a)** Control group x200. **(b)** A3 mild positive in bile duct epithelium (red arrow) x200, ABC method**.**

DISCUSSION AND CONCLUSION

DEN is a carcinogenic compound that is linked to a number of industries and their products. It has evenbeen reported that some therapeutic drugs can occur during metabolism in the liver (Goodsell, 2004; Straif et al., 2000). DEN is removed by enzymes of the cytochrome P450 bound to the monooxygenase system, where reactive intermediates can cause necrosis, mutation and cancer if they are not being excreted in the urine and are instead forming covalent bonds with important cell components, since they have little interest in the catalytic regions of binding enzymes (Chiarello et al., 1998).

In histopathological studies previously performed in fish (especially zebrafish or medaka), multifocal or diffuse vacuolization in hepatocytes, coagulative necrosis, spongiosis, hepatic cysts, hyalinization in the hepatocytes, mononuclear inflammatory reaction, non-neoplastic proliferative changes, cholangiofibrosis, bile duct epithelium hyperplasia, hepatocellular adenoma or carcinoma, and cholangioma or cholangiocarcinoma were encountered (Boorman et al., 1997). In a study conducted in Medekas, samples were taken on days 3, 8, 14 and 21, and degenerative and necrotic changes were observed in the histopathological examination, while neoplastic changes were not observed (Braunbeck et al., 1992). In this study, the findings of hyperaemia, degeneration, mononuclear cell infiltration, necrosis, bile duct epithelium hyperplasia, cholangiofibrosis and fatty degeneration in the liver tissue can be found. However, spongiosis, hepatic cyst, non-neoplastic proliferative changes, hepatocellular adenoma or carcinoma, cholangioma or cholangiocarcinoma were not observed. In this study, Although doses similar to those used in previous studies were used in this study, it was thought that the reason why no tumors were formed was due to the shorter autopsy times, as in the study by Braunbeck et al (1992). In our study necropsy examinations were performed at 15^{th}, 30^{th} and 45^{th} days while in other studies they were performed between 24^{th} and 36^{th} weeks (Mizgireuv et al., 2004). In another study by Machada et al. (2014), necropsies were performed at 3, 6 and 9 months following DEN application, and preneoplastic lesions were found at 6 and 9 months trials. While DEN was thought to form tumours in the body

after longer periods of time, we suggest that rainbow trout were more resistant to environmental factors than other fish used in laboratory experiments.

Cytochrome P450 reductase is a family of haemoproteins found in many mammals and insects. It is found in hepatocytes, especially in the centrilobular region cells. The cytochrome P450 reductase enzyme plays a role in the biotransformation of drugs and chemicals (pesticides, polyaromatic hydrocarbons, etc.) and draws attention, as it is an important source of reactive oxygen species. Many pesticide classes (including polychlorlubiphenyls and cyclodienes, such as DDT, aldrin/dieldrin, chlorine, toxafene, heptachlor, lindane, endosulfan and myrex) induce cytochrome P450 reductase. GST is a family of isoenzymes that play an important role in protecting cells against cytotoxic and carcinogenic agents. Pesticide metabolites are rendered inactive by conjugation with GSH. The ability of pesticides to bind to the "mu" isoenzyme of the GST enzyme is high (Kurutas et al., 2003).

As a result of immunohistochemical staining, in C2, A3, B3 and C3 groups in terms of CYP1A, mild positive in bile duct epithelium and negative in other groups; all groups were negative staining in terms of cytokeratin 18 and for GST, the mild positive in the bile duct epithelium in the A3, B3 and C3 groups, and negative in the other groups. The negative results in the immunohistochemical staining could be attributed to the absence of neoplastic changes in the cells. In addition, the levels of these enzymes in the blood have been determined in fish, following DEN administration, but have not been studied immunohistochemically in other tissues. Therefore, no comparison was made in terms of immunohistochemical findings.

As a result, DEN is toxic and carcinogenic to all living creatures that come into contact with this substance, as in fish. With this study, it was shown that diethylnitrosamine causes significant damage to the liver tissue even in short-term applications in fish.

ACKNOWLEDGEMENTS

This research was supported by the Research Fund of Ondokuz Mayis University (Project Number: PYO.VET.1901.15.001).

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