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Protective Effect of Silymarin on Diethylnitrosamine Induced Hepatocellular Carcinoma Model in Wistar Rats; Cancer Patterns and Cytological Variants*

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Abstract: In this study, the protective effect of silymarin was investigated with histopathological methods in terms of cancer patterns and cytological variants in the hepatocellular carcinoma model created by applying diethylnitrosamine experimentally in rats. For this purpose, the rats were randomly divided into 5 groups. The rats in the control group were fed pellet feed and drinking water for 20 weeks. Animals in Sham group were administered with propylene glycol dissolved in 0.9% NaCl intraperitoneally 3 times a week for 20 weeks. Animals in DEN group administered DEN at a dose of 50 mg/kg once a week for 20 weeks. The rats in the DEN +Silymarin group were Silymarin intraperitoneally administered for 21 weeks 3 times a week at a dose of 100 mg/kg, starting 1 week prior to the administration of DEN. Silymarin was administered to rats in the group of Silymarin 3 times a week intraperitoneally for 20 weeks at a dose of 100 mg/kg. At the end of the study, systemic necropsy of the animals was performed and liver tissue samples were taken. After routine tissue procedures, sections were examined under the light microscope. We observed that Hepatocellular carcinoma occurred in DEN and DEN + Silymarin groups.

Keywords: Diethylnitrosamine, Hepatocellular Carcinoma, Patterns, Silymarin, Variants.

Wistar Ratlarında Dietilnitrozamin Uygulanarak Oluşturulan Hepatoselüler Karsinom Modelinde Silimarin'in Koruyucu Etkisi; Kanser Paternleri ve Sitolojik Varyantlar

Öz: Bu çalışmada, ratlarda deneysel olarak Dietilnitrozamin uygulanarak oluşturulan Hepatoselüler karsinom modelinde Silimarin'in koruyucu etkisi kanser paternleri ve sitolojik varyantlar açısından histopatolojik metodlarla araştırılmıştır. Bu amaçla ratlar 5 gruba ayrıldı. Kontrol grubundaki ratlara 20 hafta boyunca pelet yem ve içme suyu verildi. Sham grubundaki hayvanlara %0.9 NaCl içerisinde çözdürülmüş propilen glikol intraperitoneal yolla haftada 3 kez olmak üzere 20 hafta boyunca verildi. DEN grubundaki hayvanlara 50 mg/kg dozunda haftada 1 kez olmak üzere 20 hafta boyunca DEN uygulaması yapıldı. DEN+Silimarin grubundaki ratlara DEN uygulamasından 1 hafta önce başlayacak şekilde 100 mg/kg dozunda haftada 3 kez olmak üzere 21 hafta boyunca intraperitoneal yolla Silimarin uygulandı. Silimarin grubundaki ratlara 100 mg/kg dozunda 20 hafta boyunca intraperitoneal yolla haftada 3 kez olmak üzere Silimarin uygulandı. Çalışmanın sonunda, hayvanların sistemik nekropsisi yapıldı ve karaciğer doku örnekleri alındı. Rutin doku takip işlemlerinden sonra kesitler ışık mikroskobu altında değerlendirildi. DEN ve DEN+Silimarin gruplarında Hepatoselüler karsinom oluştuğunu gözlemledik.

Anahtar Kelimeler: Dietilnitrozamin, Hepatoselüler Karsinom, Paternler, Silimarin, Varyantlar.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy, describe as the second and sixth cancer-related deaths in men and women in the world (1,2). The prognosis of HCC is poor and the five-year survival rate is below 16% (3,4). The incidence of HCC with high mortality is primarily due to diagnosed in advanced stages (5). The prevalence of HCC is increasing in various regions of the world. HCC has higher prevalance in Africa (sub-Saharan) and Asia (Eastern) regions where the hepatitis B virus infection is endemic; it also has increasing prevalence in Western countries (North and South Europe, North America) due to the increasing averages of alcoholic liver disease and hepatitis C infection (6).

Diethylnitrosamine (DEN) is well known as strong hepatocarcinogen and hepatotoxin, is synthesized endogenously and located in agricultural chemicals, cosmetics, pharmaceutical preparations, tobacco smoke, soybean, cheese and food preservatives (7,8). Silymarin is a polyphenolic flavonoid derived from the seeds and fruits of the Silybum marianum L. (Milk thistle) has been used in the treatment of various diseases as diabetes, cancer, hepatitis and liver cirrhosis (9,10).

In our study, we aimed to research the role of DEN in the rat HCC model, and evaluate in detail the anticarcinogenic effect of Silymarin by histopathological methods in terms of cancer patterns and cytological variants.

MATERIALS and METHODS

Animals and Experimental Design

The study was performed on fifty male Wistar-Albino rats. Animals were obtained from the Erzurum Veterinary Control Institute Experimental Animals Unit. Standard temperature (23 ± 2 °C), humidity (%55) and light/dark conditions (12-hour day-night cycle) were provided for the rats. They were hosted in the Kafkas University Experimental Animals in the Application and Research Center Laboratory. During the trial, the animals were given standart feed and water ad libitum. The ethics committee report of the study was obtained from Kafkas University Animal Experimentals Local Ethics Committee (Authorization number: KAU-HADYEK-2015-095, Date: 17.12.2015). The rats were randomly divided into 5 groups. 1. Control Group (n=10): Pellet feed and drinking water were given for 20 weeks. 2. Sham Group (n=10): Propylene glycol (75/25) dissolved in 0.9% NaCl was administered intraperitoneally three times a week. 3. Silymarin Group (n=10): Silymarin was administered as a dose of 100 mg/kg intraperitoneally three times a week for 20 weeks. 4. DEN Group (n=10): DEN was administered as a dose of 50 mg/kg intraperitoneally once a week for 20 weeks. 5. DEN + Silymarin Group (n=10): DEN was administered as a dose of 50 mg/kg intraperitoneally once a week for 20 weeks. Silymarin was administered as a dose of 100 mg/kg intraperitoneally administered intraperitoneally for 21 weeks, three times a week, starting 1 week before DEN administration. At the end of the study, systemic necropsy was performed on rats under pentobarbital anesthesia.

Histopathological Investigations

Liver samples from rats were fixed in 10 % buffered formaldehyde solution. After routine tissue procedures, paraffin blocks were prepared and sections with a thickness of 5 µm were taken for Hematoxylin Eosin (H&E) staining. Sections were examined with H&E in the light microscope to determine the histopathological patterns & cytological variants and photographed with Cell ^P Program.

RESULTS

In the histopathological examination of Control (Fig. 1A), Sham (Fig. 1B) and Silymarin Group (Fig. 1C) livers; we observed that the liver tissue retained its normal lobular and sinusoidal structure, hepatocyte plates had a proper arrangement. Hepatocytes were

detected to be similar in size and shape. HCC was observed in the animals of the DEN group. Trabecular and acinar pattern were detected as dominant in tumor tissue. In some areas only, trabecular structures were observed (Fig. 2A), whereas in some areas only acinar structures were present (Fig. 2B). There were also areas where these two patterns are mixed (Fig. 2C). The trabecular structures were 3-10 cells thick and were separated by sinusoid-like spaces that were placed by endothelial cells. Lightly eosinophilic stained content was rarely present in lumen in pseudoglandular structures consisting in acinar pattern. Tumor cells showed morphological similarity to normal hepatocytes and it was observed that the differentiation was well. In some areas, it was determined that the hepatocytes were in cuboidal even columnar shapes (Fig. 2D). The cytoplasm of tumor cells was observed to be eosinophilic. The nucleus / cytoplasm ratio increased in favor of the nucleus. Nucleus showed atypical character. It was determined that the nuclei could be round, oval or irregular and showed hyperchromasia. In some areas, the nucleoli were eosinophilic, single and prominent, while in some areas the number was increased. In addition, large areas of liver tissue surrounded by fibrous capsule and filled with blood in the liver, bile duct hyperplasia, large and small vacuoles in the form of fat degeneration, increased connective tissue, bile pigment, cells with transparent cytoplasm (clear cells) (Fig. 2E), pale bodies (Fig. 2F) and hyaline droplets (Fig. 2G) were also histopathological changes observed in the tumor tissue. Although mitosis was observed in a large number in some areas, it was negligible in some areas (Fig. 2H). In rats administered with DEN+Silymarin, HCC was diagnosed. In some areas only, trabecular structures were observed (Fig. 3A), whereas in some areas only acinar structures (Fig. 3B) were present. Similar to the DEN group, the common histopathological pattern was a mix pattern with trabecular and acinar structures (Fig. 3C). In addition; different degrees of atypia (Fig. 3D), nuclear irregularity, increased nucleus-cytoplasm ratio, hyperchromasia, cavernous areas filled with blood, bile duct hyperplasia, fat vacuoles in hepatocytes, increased connective tissue, bile pigment, clear cells (Fig. 3E), pale bodies (Fig. 3F), hyaline degeneration (Fig. 3G) and mitotic figures (Fig. 3H) were observed in tumor tissue.

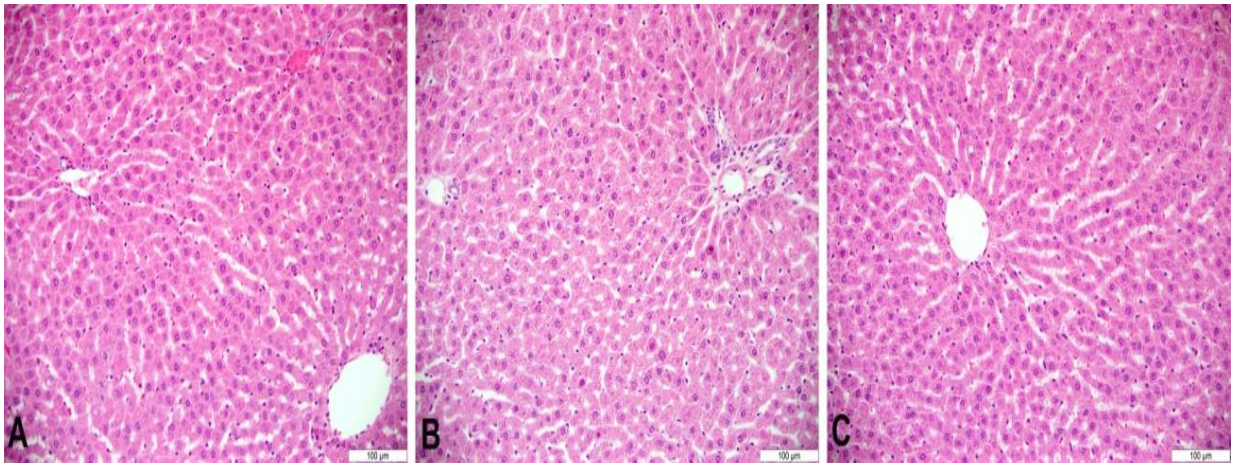

Figure 1. Control Group, H&E (A), Sham Group (B), Silymarin Group (C).
Şekil 1. Kontrol Grubu, H&E, (A), Sham Grubu (B), Silimarin Grubu (C).

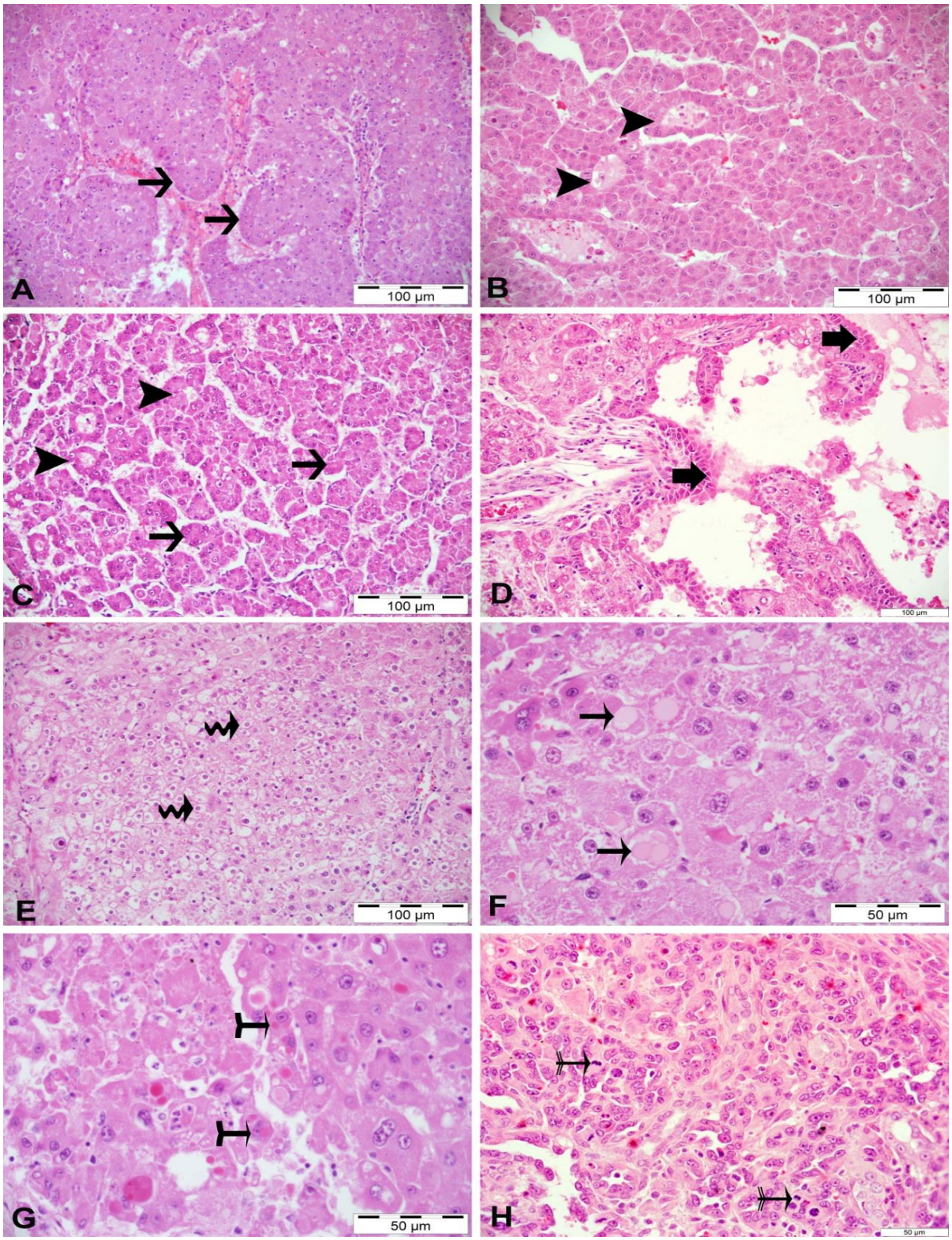

Figure 2. DEN Group, H&E, trabecular (arrows) structures (A), acinar (arrowheads) structures (B), trabecular (arrows) and acinar (arrowheads) structures (C), atypical tumor cells (arrows) (D), clear cells (arrows) (E), pale bodies (arrows) (F), hyaline droplets (arrows) (G), mitotic figures (arrows) (H).

Şekil 2. DEN Grubu, H&E, trabeküler (oklar) yapılar (A), asiner (ok başları) yapılar (B), trabeküler (oklar) ve asiner (okbaşları) yapılar (C), atipik tumor hücreleri (oklar) (D), şeffaf hücreler (oklar) (E), soluk cisimcikler (oklar) (F), hiyalin damlacıkları (oklar) (G), mitotik figürler (oklar) (H).

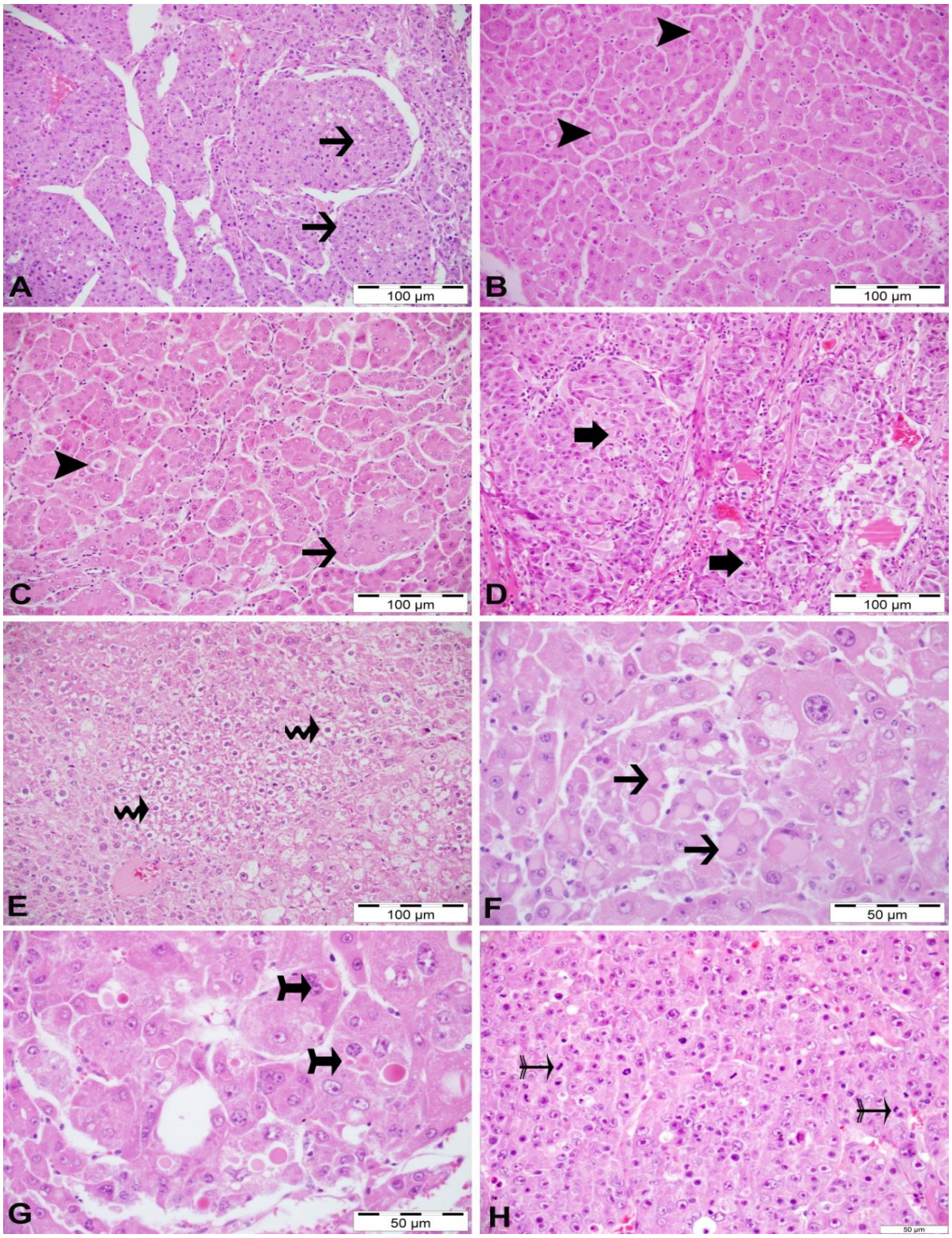

Figure 3. DEN+Silymarin group, H&E, trabecular structures (arrows) (A), acinar structures (arrowsheads) (B), trabecular (arrow) and acinar (arrowhead) structures (C), pleomorphism in tumor cells (arrows) (D), clear cells (arrows) (E), pale bodies (arrows) (F), hyaline droplets (arrows) (G), mitotic figures (arrows) (H).
Şekil 3. DEN+Silimarin Grubu, H&E, trabeküler yapılar (oklar) (A), asiner yapılar (okbaşları) (B), trabeküler (ok) ve asiner (okbaşı) yapılar (C), tümör hücrelerinde pleomorfizm (oklar) (D), şeffaf hücreler (oklar) (E), soluk cisimcikler (oklar) (F), hiyalin damlacıkları (oklar) (G), mitotik figürler (oklar) (H).

DISCUSSION and CONCLUSION

Four histopathological patterns exist in HCC. These are trabecular, pseudoglandular, solid and cirrhotic patterns (11). The trabecular pattern is the most common pattern and is also referred to as sinusoidal pattern (12). In this pattern, the cell layers are 3 or more cells thick (13). Thickness of even 5 to 10 cells, seldomly up to 20 cell-layered trabecules may occur (11). The acinar pattern of HCC is also referred to as pseudoglandular or adenoid pattern. It is less common than the trabecular pattern. The pseudoglandular pattern is characterized by gland-like structures and acini (14). The acinar pattern can often be seen as a mix in the trabecular pattern (15). Solid pattern; neoplastic hepatocytes form a solid mass lack of sinusoids and is characterized by dense tumor cell aggregates (11,16). It is typically seen as a loss of the reticulin framework (14). Cirrhotic type ise rarely seen (15). Prominent fibrosis is present along the sinusoidal cavities and at varying degrees of atrophy are seen in trabeculae formed by tumor cells (17). It can accompany any of the other patterns (14). Tumor cells in HCCs are generally polygonal, but may be cuboidal or columnar. It has thin granular eosinophilic cytoplasm. In well and moderately degree differentiated tumors, bile canaliculi are easily seen, but not in high-grade HCCs. An increase in the nucleus-cytoplasm ratio is observed. The nucleus is round-oval and contains a distinct nuclei and the nucleus membrane is irregular. Even though the intranuclear cytoplasmic invaginations are not a specific finding and they are commonly seen. Various cytoplasmic inclusions can be detected in tumor cells. Fat droplets occur in two-thirds of tumors. Diffuse accumulation of fat or glycogen causes the cytoplasm to appear transparent. Because of these changes, the tumor is called clear cell carcinoma. Mallory hyaline is present in approximately 20% of cases and refers to the clustering of intermediate filaments (18). Globular proteinous eosinophilic inclusions occur in 20% of cases. The light-colored inclusions, which are observed in 8% of cases, are called pale bodies and express fibrinogen accumulation. Copper binding protein or copper was detected in 28% of HCCs and associated with the presence of bile in the tumor (13).

As previously reported, HCC (19-27) and cholangioadenocarcinoma (28) were induced by DEN administration. Histopathological alterations in these studies briefly; liver lobular structure disruption (21,22), trabecular structures formation (19), atypia in the cells (19,21,23,24), differences in the nucleus-cytoplasm ratio (23,24), hyperchromasia (20-22), megalocytosis and foam cytoplasm cell appearance (20), mitotic figures (20,22,23), tumor cells with granular cytoplasm (21,22) multi-nuclei bizarre giant cells (24), hyaline globules (21), apoptosis (20), bile duct proliferation (21) and inflammatory cell infiltration (21). Similar to these studies (19-27) we demonstrated that HCC was occurred in the DEN group however, the dominant histopathological pattern was a mixture containing trabecular and acinar structures. In tumor tissue; atypia, differences in nucleus/cytoplasm ratio, hyperchromasia, mitosis, fatty changes, hyaline droplets, pale bodies, wide hemorrhage areas surrounded by fibrous capsules, bile duct hyperplasia and clear cells were also observed. According to our data, DEN was found to be very useful in inducing an experimental liver cancer model especially HCC.

Recent studies show that Silymarin has a histopathologically protective effect against liver injury and cancer as a result of DEN administration (19-21,29,30). In DEN+Silymarin group similar to the DEN group, a mix pattern of trabecular and acinar structures of HCC was observed. In the histopathological examinations, atypia, hyperchromasia, mitosis and fatty changes were observed in the cells in the tumor area. In addition, hyaline droplets, pale bodies, wide areas of hemorrhage surrounded by fibrous capsules, bile duct hyperplasia and clear cells in the tumor area were also detected. In the light of these findings, we determined that Silymarin didn’t show any anticarcinogenic effect in contrast to the literature data (19-21,29,30).

In conclusion, we thought that 20 weeks of DEN administration is very effective in inducing HCC. In addition, we revealed histopathologically in detail that Silimarin has no protective effect against DEN administration. We suggest that the gavage route should be preferred in order for Silimarin to have a stronger protective effect.

Conflict of interest

The authors declare that they have no conflict of interest.

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