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EFFECT ON ENDOPLASMIC RETICULUM STRESS OF THE COMBINED ORAL CONTRACEPTIVES IN THE LIVER

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Abstract

Objective: We aimed to evaluate the effects of combined oral contraceptive active ingredients ethinylestradiol, drospirenone, and ethinylestradiol+drospirenone for histopathological changes, and endoplasmic reticulum stress levels in the liver.

Methods: In the study, 37 to 8-week-old Balb/c female mice were used. Mice were randomly divided into the control, sham, ethinylestradiol, drospirenone, and ethinylestradiol+drospirenone groups. Experimental groups were administered ethinylestradiol, drospirenone, and ethinylestradiol+drospirenone with gavage for 35 days. In liver tissue sections, histopathological changes were detected with hematoxylin&eosin, orcein, Mallory's Azan, and periodic acid-Schiff, and the presence of endoplasmic reticulum stress was detected by Chop and Grp78 immunostaining.

Results: The ethinylestradiol+drospirenone group showed significant histopathological changes compared to the control group. Some degenerative changes were noted such as swelling and size differences in hepatocytes in the ethinylestradiol+drospirenone group. When compared to the control group, an increased collagen and elastic fibers density around the vena centralis was observed in the ethinylestradiol+drospirenone group. The expression level of Grp78 protein in female mice given ethinylestradiol+drospirenone was statistically significantly increased compared to the control group. The expression level of Chop protein was significantly increased in the ethinylestradiol, drospirenone, and ethinylestradiol+drospirenone groups.

Conclusion: We concluded that the use of combined oral contraceptives increases endoplasmic reticulum stress in mouse liver tissue, and as a result, it may cause liver histopathological disorders by promoting cell death.

Keywords: *Combined oral contraceptive, drospirenone, ethinylestradiol, liver, Grp78, Chop*.

Introduction

Combined oral contraceptives (COCs) which contain 17-ethinyl estradiol (EE) as an estrogen component and drospirenone (DRSP) containing progestogen have been preferred not only for the prevention of pregnancy but also for menstrual cycle irregularities, relief of postmenopausal symptoms, and various acne problems in women.[1, 2] The novel progestins with high specificity have been designed to avoid interaction with other receptors and prevent androgenic, estrogenic, or glucocorticoid-related side effects.[3] In addition to being used for therapeutic purposes, COCs make positive contributions to health such as reducing the risk of some types of cancer such as ovarian and endometrial cancer, reducing the risk of rheumatoid arthritis, preventing ectopic pregnancy, and increasing insulin sensitivity.[4, 5] However, COCs have also caused various side effects such as depression, migraine headaches, breast and cervical cancer, stroke, cardiovascular diseases, venous thromboembolism and high systolic blood pressure, inflammatory bowel disease, benign or malignant liver tumors, and obesity.[6-12]

The liver is one of the main secretory organs that play a fundamental role in carbohydrate, protein, and lipid metabolism. Liver hepatocyte cells are rich in the endoplasmic reticulum (ER). It is known that this organelle is associated with plasma proteins, glycogen and lipid synthesis, and detoxification mechanisms in the liver.[13, 14] The ER in hepatocytes has a notable capacity to maintain extracellular and intracellular balance by maintaining vital hepatic metabolic functions.[15, 16] Hepatic viral infections, gene mutations, metabolic disorders, and extreme use of ethanol or drugs can induce ER stress (ERS). Hepatocyte cells cope with ER stress through an adaptive response named unfolded protein response (UPR), which obtained enhancing protein folding and degradation in the ER and down-regulating overall protein synthesis.[17-19] In response to ER stress, main pathways are activated, which in turn, mediate the UPR.[20, 21] The Glucose-regulated protein of 78 kD (Grp78) is chiefly ER chaperone protein and it is critical for protein quality control of the ER, as well as a master regulator of the UPR. Grp78 is involved in the activation of IRE (the type-I ER transmembrane protein Kinase), PERK (protein kinase RNA-like ER kinase), and ATF-6 (ATF-6 N terminal domain) pathways as well as in the establishment of the ERS response.[22] Under ERS, Grp78 releases and activates unfolded protein response sensors to restore ER homeostasis. In response to prolonged and severe ER stress, the UPR triggers apoptotic pathways that lead to cell death.[23] C/EBP-homologous protein (Chop), a proapoptotic transcription factor, is activated in ER stress-mediated apoptosis.[24]

This study aims to examine the effects of ethinyl estradiol and drospirenone active ingredients and combined oral contraceptive forms on histopathological and ERS in the liver.

Methods

In our study, 37 Balb/c female mice (weighing 20±25 g, 6-8 weeks old) were provided from the Akdeniz University Experimental Animals Research and Application Center. Each of the mice was kept in standard laboratory conditions which were without water and food restrictions; 12 hours light/12 hours dark cycle. The Institutional Animal Ethical Committee of Akdeniz University (Antalya, Turkey) approved the study. (reference number: 2022.01.009).

The mice were randomly divided into five groups such as; EE Group (n:9), DRSP Group (n:9), EE+DRSP Group (n:9), Sham Group (n:5), and Control Group (n:5). Female mice were given 60 µg of DRSP for DRSP group and 0.6 µg of EE for the EE group by gavage every day for 35 days.[25] EE and DRSP were dissolved in 100% Ethanol. Ethanol-EE containing DRSP was mixed with sesame oil. The sesame oil-ethanol mixture was kept in an incubator at 37° for 24 hours for evaporation of the ethanol.[26] Yasmin® tablets (Germany) were dissolved in water and administered to the EE+DRSP group by gavage for 35 days. To the Sham group, sesame oil with evaporated ethanol was given by gavage for 35 days. Nothing was administered to the Control group.

At the end of 35 days, the mice were anesthetized with ketamine (100 mg/kg; Alfasan) + xylazine hydrochloride (10 mg/kg; Bayer). Mice were sacrificed by cervical dislocation and liver tissues were taken. Samples were fixed in 4% paraformaldehyde for 24 h and then were washed in running water for 4 h. The samples were dehydrated through graded alcohols and embedded in paraplast.

Histopathological Staining

Five-micrometer of liver sections were prepared. The sections were deparaffinized and rehydrated. Liver histopathology was evaluated via Hematoxylin&Eosin (H&E) staining and also preferred to stain with Mallory's Azan for collagen fibers, Orcein for elastic fibers, and Periodic Acid Schiff (PAS) to show glycogen content in hepatocytes. Taken sections were dehydrated for 5 min. in the increasing alcohol series, and slides were covered with entellan after using the xylol twice for 10 minutes. They were evaluated using a bright-field microscope and photographed.

Immunohistochemical Staining

Streptavidin-biotin peroxidase method was used for immunohistochemical staining method. Liver tissues were dewaxed with xylene and rehydrated in graded alcohol and washed with deionized water. 0.1 M sodium citrate was used for Antigen retrieval. The sections were washed using Tris-buffered saline (TBS). Endogenous peroxidase activity of tissues was blocked by incubating with 3% hydrogen peroxide (H_2O_2) for 20 min. Serum blocking was applied using 5% normal goat serum for 30 minutes at room temperature. Primary antibodies (Grp78-ab109659, 1:200 and Chop-ab63392, 1:200) incubated at + 4°C overnight. After washing in TBS, sections were incubated with a secondary antibody (Cell Signaling, 8114S) for 30 min. Diamino benzidine tetrachloride (DAB) was used as the chromogen. The sections were counterstained using Mayer hematoxylin. Then, they were respectively taken into distilled water, increasing alcohol series, and xylol. Then the slides were covered using entellan. The preparations were evaluated using a bright-field microscope and photographed.

Statistical Analysis

The slides were examined using an axioplan microscope (Zeiss, Germany) and photographed. Then, staining intensities were evaluated quantitatively with the Image J program. The data obtained were analyzed using the GraphPad (Prisms10) program using post hoc Bonferroni test and One Way ANOVA tests to determine statistical differences between groups.The results were presented as

mean ± standard error of the mean (SEM); $p<0.05$ between different groups were considered statistically significant.

Results

Histopathological Findings

The histopathological differences between the experimental groups as a result of H&E staining in the liver were shown in Figure 1.

The control group had a classical liver cell structure that has radially arranged hepatocyte cords around the central vein, and normal sinusoidal spaces between the cords. The arrangement of the cords in the EE+DRSP group was impaired (Figure 1A, asterisk). Some degenerative changes were noted such as swelling and size differences in hepatocytes (Figure 1A, thick arrow). Hepatocytes, whose cytoplasmic borders could not be clearly distinguished, did not have classical polygonal shapes (Figure 1A, thin arrow). Compared to the control group, hepatocyte cytoplasms showed paler staining in the EE+DRSP group. No pathological changes in liver histology were determined in the other groups.

The liver connective tissue collagen fiber density was shown via Mallory's Azan staining. An increased collagen fiber density around the vena centralis was observed in the EE+DRSP group compared to the control (Figure 1B, arrow).

Elastic fiber density was detected to increase around the vena centralis in the EE+DRSP, EE, and DRSP groups compared to the control and sham groups using Orcein staining (Figure 1C, arrows).

In the PAS staining method results, it was seen that the glycogen density in the hepatocyte cytoplasm of the EE+DRSP group was low in terms of glycogen distribution in hepatocytes. (Figure 1D, arrow).

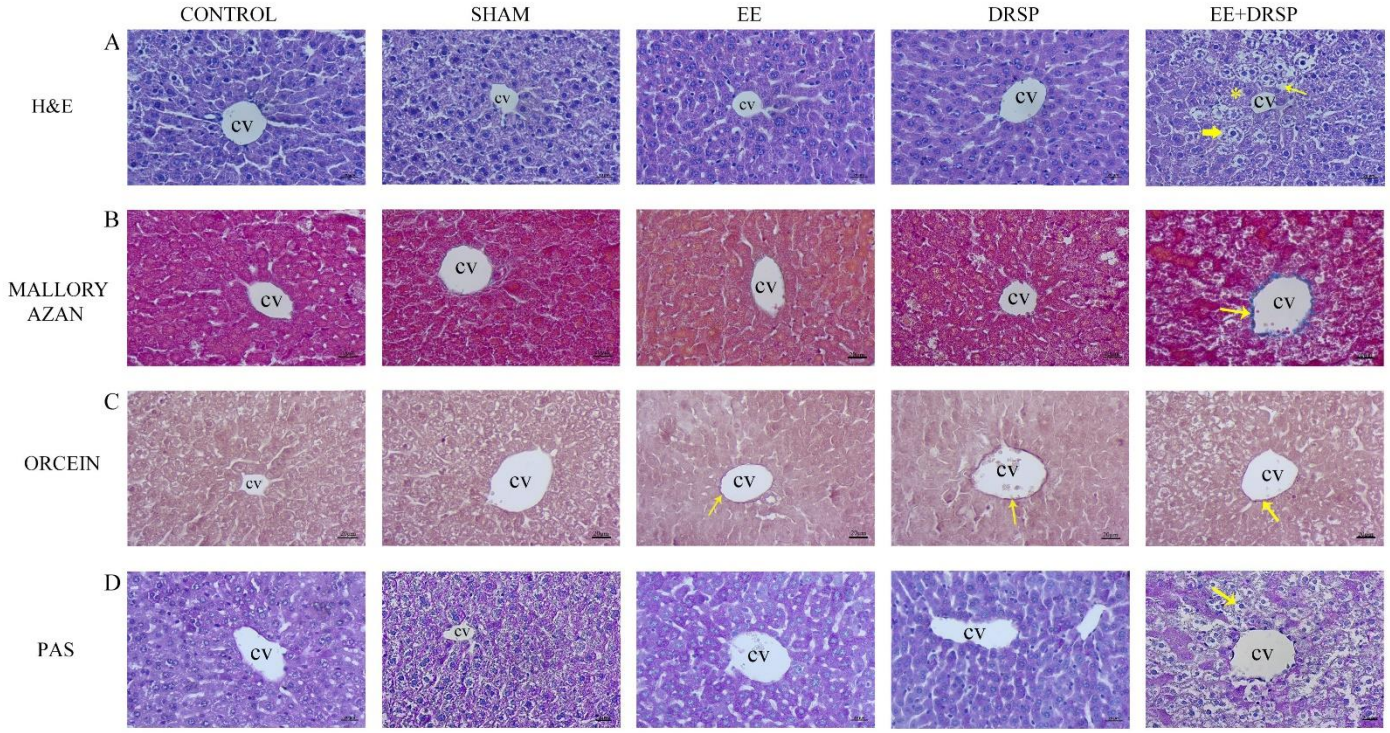

Figure 1. Histological structure and collagen, elastic fiber, and glycogen changes of liver tissue in Control, Sham, EE, DRSP, and EE+DRSP groups. Scale bars show 40 µm and apply to all panels. CV: Central Vein. **(A)** Representative histology images of H&E staining. Irregularities in the radial alignment of hepatocytes (*), hepatocytes that could not be preserved polygonal shapes (thin arrow) and show ballooning degeneration (thick arrow). **(B)** Mallory's Azan staining shows increased collagen fiber density around the central vein in the livers of the EE+DRSP group (arrow) compared to the control. **(C)** Orcein staining of livers. Elastic fiber density determined by Orsein staining increased in the EE, DRSP and EE+DRSP group (arrows) compared to the control group around the central vein. **(D)** PAS staining in the livers. Decreased glycogen density of liver hepatocytes in the EE+DRSP group (arrow).

Immunohistochemical Findings

The immunohistochemical staining method was used to detect the expression levels of the ER stress molecules, Grp78 and Chop, in hepatocytes. As shown in Figures 2 and 3, EE, DRSP, and EE+DRSP increased the expression levels of ER stress markers in hepatocyte cells.

While Grp78 was generally expressed in liver tissue, its expression was more intense in the cytoplasm of hepatocytes around the vena centralis, especially for the EE+DRSP group (Figure 2A). According to the H-SCORE results of the Grp78 protein, while the EE+DRSP group increased statistically significantly compared to the control group, there was no significant increase in the DRSP and EE groups (Figure 2B, $p<0.0001$.

A remarkable increase in nuclear-stained Chop expression was observed in the EE, DRSP, and EE+DRSP groups (Figure 3A).

The increase in Chop H-SCORE expression in the liver tissue of the EE, DRSP, and EE+DRSP groups was found to be statistically significant compared to the control group (Figure 3B, p:0.0049; p:0.0006; $p<0.0001$.

In conclusion, these findings show that ER stress was increased and ER-mediated apoptosis was induced in hepatocytes of the EE, DRSP, and EE+DRSP groups.

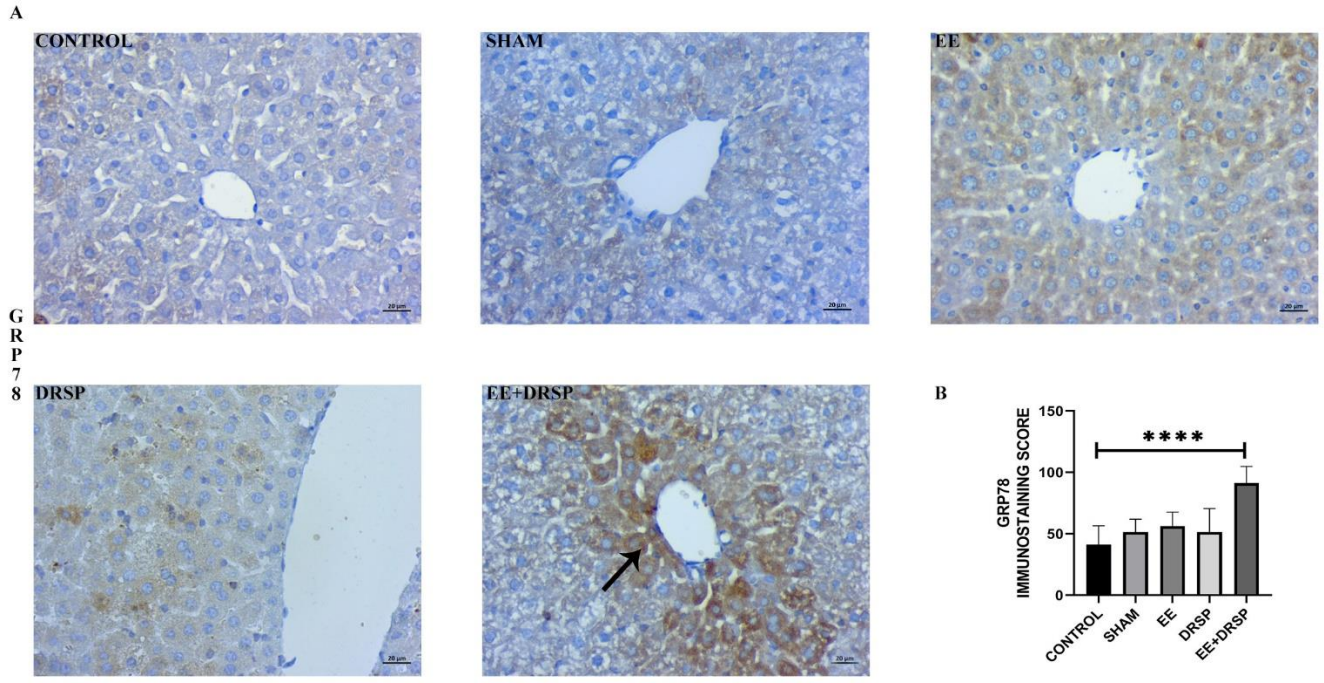

Figure 2. A. Immunohistochemical staining of ER stress markers in the liver. Representative photomicrographs showing Grp78 staining in Control, Sham, EE, DRSP, and EE+DRSP groups. The intensity of Grp78 expression around the vena center in the livers of the EE+DRSP group (arrow). **B.** Quantitative analysis of Grp78 staining. Statistical analysis was done by one-way ANOVA with all pairwise multiple comparison procedures with the Bonferroni test. Values are given as mean ± SEM. $p<0.0001$(****). While the expression level of Grp78 protein increased statistically in the EE+DRSP group compared to the Control group, there was no significant increase in the expression levels of Grp78 protein in the DRSP and EE groups.

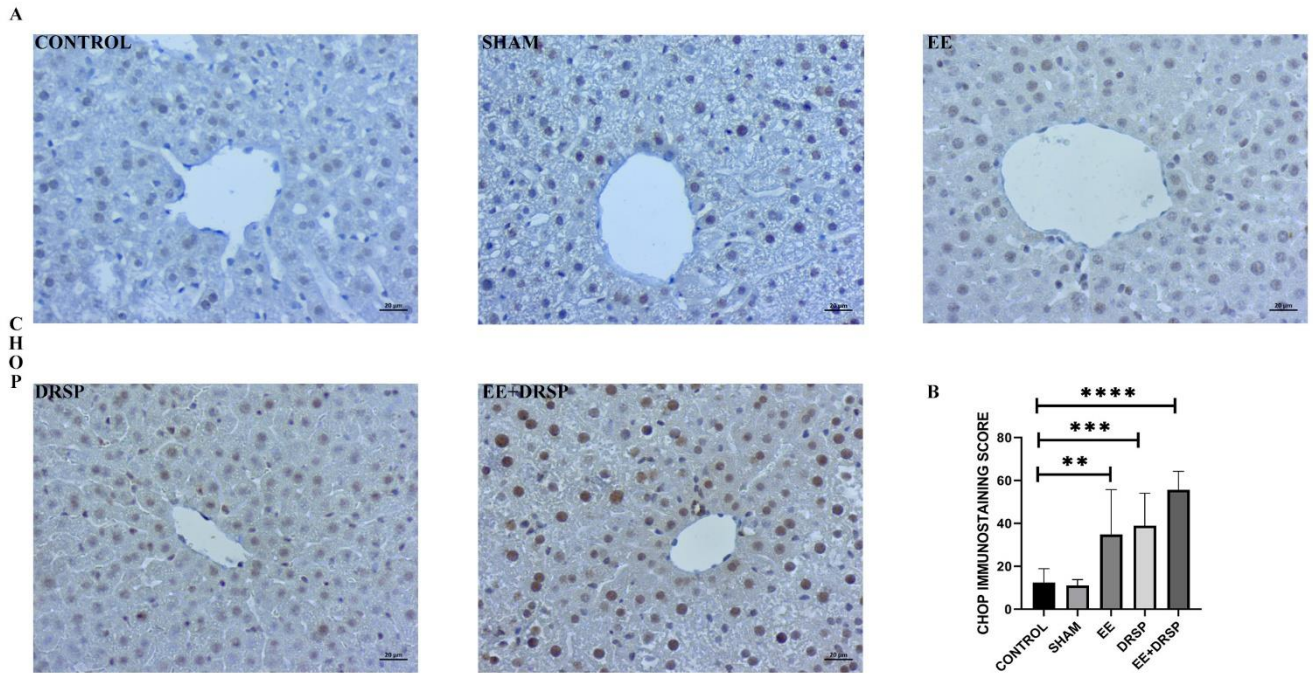

Figure 3. A. Immunohistochemical staining of ER stress markers in the liver. Representative photomicrographs showing Chop staining in Control, Sham, EE, DRSP, and EE+DRSP groups. **B.** Quantitative analysis of Chop staining. Statistical analysis was done by one-way ANOVA with all pairwise multiple comparison procedures with the Bonferroni test. Values are given as mean ± SEM. It was found that Chop expression in liver tissue of EE, DRSP, and EE+DRSP groups increased statistically significantly compared to the control group. p:0.0049(**); p:0.0006(***); $p<0.0001$(****).

Discussion

The liver is an organ that plays an important role in the biotransformation of drugs and toxins.[27] For this reason, it is the main target for caused damage by drugs. Studies have shown that various pathological conditions in the liver induce ER stress.[17-19, 28] However, it is largely unknown whether COCs have a role in the development of ER stress and pathologies in the liver. In this study, we demonstrated histopathological changes and ER stress levels in the liver tissue of mice applied EE, DRSP, and EE+DRSP.

ER is the organelle that protein synthesis, folding, maturation and transport, calcium storage, and lipid biosynthesis. ERS is defined as the balance between the protein folding capacity of the ER, and the processed protein load resulting in the accumulation of misfolded or unfolded protein.[29] The cell activates UPR pathways to counteract ER stress, which occurs as a result of the imbalance between the load of unfolded proteins in the ER and the capacity of the cellular mechanism to handle this load. Disruption of ER homeostasis contributes to hepatic steatosis, inflammation, and insulin resistance in the liver.[30] It has been reported that the UPR was activated in various liver diseases such as fatty liver disease, viral hepatitis, and alcohol-induced liver injury.[17-19, 28, 31]

The liver is surrounded by a collagen-elastic fiber–containing capsule (Glisson) and is lined by the peritoneum.[14] In a study of liver tissue of different species, elastic fibers have been seen individually and in small numbers in humans and baboons; found to be thinner but more abundant in both the portal tract and hepatic vessels in the mouse.[32] In a study by Yurdakul et al. in which liver tissues of rats in different age groups have examined, it was pointed out that the tissues of newborns were devoid of collagen fibers, and the presence of collagen fibers concentrated around the Glisson capsule, v.centralis wall and portal area of other groups. However, elastic fibers have not been found in cross-sections of any age group.[33] In rats in which liver damage has caused by cyclosporine, no damage was observed in the central vein endothelium in the elastin fiber staining and no difference was found in the glycogen distribution in the preparations stained with PAS compared to the control group.[34] In our study, as a result of our histological staining with Orcein, it was determined that the elastic fiber density around the vena centralis increased in the EE+DRSP, EE, and DRSP groups compared to the control group. In addition, as a result of our PAS histochemical staining; it was observed that glycogen storage of liver epithelial cells was weak in the EE+DRSP group, while it was normal in all other groups.

Hepatocytes have many functions, including endocrine (plasma protein secretion), exocrine (bile secretion), glucose storage (glycogen granules), and detoxification.[35] In addition, the cells around the portal and arterial vessels, which contain blood rich in oxygen, nutrients, and toxic substances in the liver, are exposed to various stress factors.[14] In a study by Klipping et al. examining the endocrine and metabolic effects of combined oral contraceptives used by healthy women aged 18-50 years, it was reported that they increased sex hormone-binding globulin and liver protein levels, and were also effective on angiotensinogen and gonadotropins.[36] A further study has shown that EE had a strong effect on liver proteins and was responsible for mild changes in pro-coagulation and fibrinolytic balance.[3] Steingold *et al.*'s study in which subjects administered oral EE revealed that circulating estrogen was more active in the liver than in other tissues such as the brain or uterus, and had a stronger effect on estrogen-dependent liver proteins than natural estradiol (E2).[37] In our study, the mice given combined oral contraceptives containing EE+DRSP showed some degenerative changes, such as swelling and size differences in hepatocytes compared to the control group. In addition, an increased collagen fiber density around the vena centralis was observed in the EE+DRSP group compared to the control.

The liver plays a major role in maintaining glucose homeostasis by regulating the absorption, accumulation, and catabolism of glucose through various metabolic signals.[38] The using COC has been associated with impaired glucose tolerance and insulin resistance, which are both risk factors for type II diabetes, and cardiovascular disease.[39] Some studies have reported that the use of COCs did not differ significantly on carbohydrate metabolism and the observed effects were not considered clinically significant.[39, 40] In our study, In our study, it was determined that glycogen distribution in hepatocytes decreased in the EE + DRSP group.

In the presence of unfolded proteins, Grp78 moves away from PERK and binds to unfolded proteins, thereby maintaining homeostasis in the ER.[41] Expression of the ERS-mediated apoptosis marker Chop is induced when the unfolded protein load in the ER cannot be balanced despite increased Grp78. Therefore, while Grp78 levels indicate ERS, Chop expression is a marker that cells can undergo ERS-mediated apoptosis.[23, 42] In stress-free conditions where ERS does not occur, Chop has low expression. IRE1, PERK, and ATF6 molecular sensors are triggered by ERS and transcriptional expression of Chop is increased. It has been reported that Chop induces apoptosis in several cell lines, while the UPR is involved in the pathogenesis of inflammation. Recent publications have shown that Chop is a key molecule not only in apoptosis but also in inflammatory responses.[43] Liver hepatocytes are rich in ER and susceptible to ER degradation and ER stress due to their synthesis and other biological functions.[14]

In one study, women using COC were associated with a higher risk of venous thromboembolism than non-using. It was also reported in this study that using EE/DRSP affected hepatic metabolism.[12] A further study has shown that EE+DRSP increases the synthesis of various liver proteins and affects lipid and carbohydrate metabolism.[36] ERS activation was observed in liver tissue in this study parallel to the aforementioned study, in which mice were given EE, DRSP, and EE+DRSP. Hepatocyte cells with apoptotic morphology and abnormalities in liver histopathology, especially observed in mice given EE+DRSP, are a sign that the cells may be prone to ERS-mediated apoptosis as a result of increased expression of ERS markers Grp78 and Chop. In contrast, a study by Taneepanichskul et al. reported that oral contraceptives did not affect heart rate, blood pressure, complete blood count, fasting plasma glucose, electrolytes, or kidney and liver functions.[44]

Previous studies have shown that natural sesame oil contains various antioxidants such as sesamolin, sesaminol,[45] tocopherol,[46] and sesamin.[47] These compounds have been documented to possess inhibitory effects on membrane lipid peroxidation and microsome peroxidation and they have been known to act as antioxidants by scavenging peroxyl radicals.[45] Studies conducted with sesame oil have reported that it had protective properties in the liver.[48-50] It has been suggested that sesame oil detoxifies the liver, reduces the

incidence of chemically induced breast tumors, eliminates hepatic dysfunction by increasing glutathione concentration,[49] and protects against oxidative stress thanks to the antioxidants it contains.[48, 50] In our study, EE and DRSP dissolved in sesame oil containing evaporated ethanol were given by gavage for 35 days. However, mice in the EE+DRSP group were given the pill dissolved in water. Based on this information, our study indicates that sesame oil used in the dissolution of ethinyl estradiol and drospirenone may have a role in suppressing the negative effects on both the histopathology of the liver and ER stress markers.

In our study, mice treated with EE+DRSP had significantly higher levels of Grp78, an ER stress marker, compared to the Control group (p<0.0001). When Chop expression levels were examined to see the apoptosis induced by ER stress, it was observed that the EE, DRSP, and EE+DRSP groups increased significantly compared to the Control and Sham groups. All these findings show that the EE+DRSP application triggers ER stress. Use of EE, DRSP, and EE+DRSP triggered Chop-mediated apoptosis. It has also been found to increase the production of connective tissue due to collagen production. In addition, we can say that it causes a change in the amount of glycogen in hepatocytes.

The structural and functional unit of the liver is the hepatic lobule. This structure is important in terms of explaining degeneration, regeneration, perfusion, and toxic effects of some substances. The region, formed by hepatocytes around the vena centralis in the portal acini model, is the weakest oxygen concentration. And this region has a role in detoxification. It is the preferred area of glycolysis, lipid formation, and drug biotransformations and is the first hepatocyte to undergo fat deposition and ischemic necrosis. Hepatocytes are susceptible to damage caused by hypoxia.[14] We predict that better histopathological findings around the central vein in the EE and DRSP groups compared to the EE+DRSP group may be due to the antioxidant property of sesame oil used as a solvent.

In conclusion, we demonstrated the activation of ER stress in hepatocytes after COC use. Time-dependent induction of these two ERS proteins may be associated with cell death, as Grp78 may be an early adaptive or protective response, while Chop is known to have a proapoptotic function. ER stress may cause liver histopathological disorders by promoting cell death due to COC.

The mechanism underlying the hepatic side effects of COC has not been extensively studied. The findings show that ethinyl estradiol and drospirenone and combined administration increase ER stress. More comprehensive studies are needed for the results of this effect of the long-term use of COCs on the ERS.

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Conflict of interest

The authors related to this article declare no conflict of interest.

Compliance with Ethical Statement

The Institutional Animal Ethical Committee of Akdeniz University (Antalya, Turkey) approved the study. (Authorization reference number: 2022.01.009).

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Author's Contributions

AC, EK: performed the Combined Oral Contraceptives Method on mice; ST: collected samples, took sections from paraffin blocks, did histological and immunohistochemical staining, and also did the statistical analysis; EK: created the project and assisted with the experimental model, optimizing experiments and data interpretation; ST, EK: wrote the manuscript.

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