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RESEARCH ARTICLE

Effect on Endoplasmic Reticulum Stress of the Combined Oral Contraceptives in the Kidney

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Abstract

Objective: This study aimed to analyze the effects of combined oral contraceptive active ingredients, Drospirenone, Ethinyl Estradiol, and Ethinyl Estradiol+Drospirenone, on liver histopathological changes and endoplasmic reticulum stress levels.

Material and Methods: In the study, 37 Balb/c female mice were used. Mice were randomly divided into the Control, Sham, Drospirenone, Ethinyl Estradiol, and Ethinyl Estradiol+Drospirenone groups. The experimental groups were administered with gavage to 8-week-old female mice for 35 days. Kidney tissue sections were applied with Hematoxylin&Eosin, Orcein, Mallory's Azan, and Periodic Acid-Schiff to detect histopathological changes, and Chop and Grp78 were used to detect Endoplasm Reticulum Stress.

Results: Significant loss of microvilli and a decrease in glycogen accumulation were observed in the apical part of some of the proximal tubules of animals in the Drospirenone and Ethinyl Estradiol+Drospirenone groups. The amount of collagen fiber stained with Mallory's Azan increased in the parietal layer of Bowman's capsule of the kidney tissues of the Drospirenone and Ethinyl Estradiol+Drospirenone applied groups, but no difference was observed in elastic fibers in all groups. The expression level of Grp78 and Chop proteins in the kidney tubules of female mice given Drospirenone, Ethinyl Estradiol, and Ethinyl Estradiol+Drospirenone was significantly higher compared to the control group.

Conclusion: In this study, it was shown that the expression of Grp78 and Chop markers detected in the mouse kidney increased as a result of Drospirenone, Ethinyl Estradiol and Ethinyl Estradiol + Drospirenone administration, thus causing kidney cell apoptosis by inducing ER-dependent death pathway activity.

Keywords: Combined oral contraceptive, drospirenone, ethinylestradiol, kidney, Grp78, Chop

INTRODUCTION

Combined oral contraceptives (COCs), which contain 17-ethinyl estradiol (EE) as an estrogen component and drospirenone (DRSP) as the progestogen, are preferred not only for preventing pregnancy but also for managing menstrual cycle irregularities, relieving postmenopausal symptoms, and addressing various acne problems in women (1). The EE in combined oral contraceptives alters certain estrogen-sensitive

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hemostatic variables and liver-produced proteins, thereby exerting estrogenic effects. DRSP, present in a combined oral contraceptive (30 mg EE/3 mg DRSP), is a 17α-spirolactone derivative with a unique pharmacological profile that combines potent progestogenic, anti-mineralocorticoid, and antiandrogenic activities (2). These novel progestins designed with high specificity, aim to avoid interactions with other receptors and prevent estrogenic, androgenic, or glucocorticoid-related side effects (3). Furthermore, studies have reported that COC use contributes positively to health by reducing the risk of certain cancers, such as ovarian and endometrial cancer, lowering the risk of rheumatoid arthritis, preventing ectopic pregnancy, and increasing insulin sensitivity (4). However, COCs may also lead to side effects such as depression (5), breast and cervical cancer (6), cardiovascular diseases, venous thromboembolism (7), high systolic blood pressure (8), changing homeostasis parameter (9), benign or malignant liver tumors (10).

The kidneys are among the organs that play a crucial role in maintaining internal balance. They regulate blood pressure, pH, renal excretion, water, and electrolyte intake to control the osmolality and volume of body fluids. Additionally, the kidneys remove waste products formed from cellular metabolism from the blood (11). The kidney contains amino acids and peptides involved in protein metabolism, and it plays essential roles in pathways such as degradation, filtration, reabsorption, and excretion (12).

The endoplasmic reticulum (ER) is an organelle that plays a vital role in protein homeostasis, including protein synthesis, folding, modification, and degradation. Under pathological conditions, Endoplasmic reticulum stress (ERS) is induced by the accumulation of unfolded or misfolded proteins in the ER lumen. Consequently, when ERS is induced, unfolded protein response (UPR) sensors serve as an adaptive response or can cause apoptosis in cells when stress is severe. The Glucose-regulated protein of 78 kD (Grp78) is a chiefly ER chaperone protein critical for protein quality control of the ER, and a master regulator of the UPR. Grp78 is included in the activation of IRE1 (the type-I ER transmembrane protein Kinase), Perk (protein kinase RNA-like ER kinase), and ATF-6 (ATF-6 N terminal domain) pathways contributing to the ERS response. Under ERS, Grp78 releases and activates unfolded protein response sensors to restore ER homeostasis. In response to prolonged and severe ERS, the UPR triggers apoptotic pathways that lead to cell death (13). C/EBP-homologous protein (Chop), a proapoptotic transcription factor, is activated in ERS -mediated apoptosis (14).

In the kidneys, various biological disturbances can induce ERS, including increased levels of protein synthesis, oxidative stress, insufficient autophagy, hypoxia, inflammatory stress, nutrient starvation, energy deprivation, cellular stress factors, and including proteostasis disorders. Adaptive responses to these stresses often utilize evolutionarily conserved biological pathways to eliminate or reduce the stress intensity that causes by protein misfolding and aggregation maintain vital functions and cellular homeostasis (15).

We aimed to determine how DRSP and EE, a commonly used combined contraceptive method in women, affect the ERS response in kidney cells, a metabolically active organ.

MATERIAL AND METHODS

In present 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 mouse was kept in standard laboratory conditions (without water and food restrictions; 12 hours light/12 hours dark cycle). The Institutional Animal Ethical Committee of Akdeniz University (Antalya, Türkiye) approved the study. (Authorization reference number: 2022.01.009).

The mice were divided into five groups; EE Group (n:9), DRSP Group (n:9), EE+DRSP Group (n:9), Sham Group (n:5), and Control Group (n:5). EE, DRSP and EE+DRSP were administered via gavage when all mice were in the meta-estrus phase. Because it was thought that during this estrous cycle phase, plasma concentrations of gonadotropins, estrogen, and progesterone would be close to those induced by oral contraception (16). 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. 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 to evaporate the alcohol. Yasmin®, Germany tablets 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 kidney tissues were taken. Tissues were fixed in 4% paraformaldehyde and then dehydrated and embedded in paraplast.

Histopathological Staining

Sections of 5µm thickness were taken, deparaffinized, and rehydrated by general protocol. The kidney sections were stained via Haematoxylin&Eosin (H&E), Mallory's Azan (MA), Orcein, and Periodic Acid Schiff (PAS). Then, the sections were taken into distilled water and dehydrated, cleared in xylene, and the slides closed with entellan. The preparations were detected using a light microscope and photographed.

Immunohistochemical Staining

Kidney tissues were dewaxed with xylene, rehydrated in graded alcohol, and washed with deionized water. Antigen retrieval was performed by incubation with 0.1 M sodium citrate (pH 6.0) at 95-100°C for 25 min. The sections were washed Tris-buffered saline (TBS) and in Tris-buffered saline-tween20 (TBS-T). The samples were blocked with 3% hydrogen peroxide (H_2O_2) for 20 min. After blocking with 5% normal goat serum for 30 minutes at room temperature before application of the primary antibody. Afterward, tissues were incubated with primary antibodies Grp78 (ab109659, 1:200) and Chop (ab63392, 1:200) at +4°C overnight. Then at room temperature, the sections were washed and incubated with secondary antibodies (Cell Signaling, 8114S) for 30 minutes. Diamino benzidine tetrachloride (DAB) was used as the chromogen. The slides were counterstained in Mayer hematoxylin. Then, the sections are taken into distilled water and dehydrated, cleared in xylene, and the slides closed with entellan. The preparations were evaluated using a bright-field microscope and photographed.

Statistical Analysis

In order to determine whether there was a statistically significant difference between the groups, the data were measured with Image J and, then post hoc Bonferroni test and One Way ANOVA tests were applied using the GraphPad (Prisms10) program. Analyses were presented as mean±standard error of the mean (SEM);p<0.05 was considered statistically different.

RESULTS

Histochemical Results

The histopathological differences between the experimental groups as a result of H&E, MA, Orcein, and PAS staining in the kidney are shown in Figure 1.

As a result of microscopic examinations, mouse kidneys in H&E-stained sections showed, that was observed typical histological structures in the control and sham groups (Figure 1A). Significant loss of microvilli in proximal tubule epithelial cells (Figure1A, arrows) and decreased glycogen accumulation in the cells of proximal convoluted tubules showed PAS-positive luminal brush border in all groups. In addition, it was revealed that tubular basement membranes showed more intense positivity with PAS staining in the EE+DRSP group compared to other groups (Figure 1D). While the amount of collagen fibers stained with MA increased in the parietal layer of Bowman's capsule of the kidney tissues of the DRSP and EE+DRSP applied groups (Figure 1B arrows), no difference was observed in elastic fibers in all groups (Figure 1C).

Immunohistochemical Results

We evaluated the ERS levels in the kidney tissue by examining the expression of two UPR molecules, Grp78 and Chop, by immunohistochemical staining.

As shown in Figures 2 and 3, EE, DRSP, and EE+DRSP treatment increased the expression levels of ERS markers. As shown in Figure 2A and B, the immunohistochemistry study reported that Grp78 was plentifully expressed in the tubules from the EE, DRSP, and EE+DRSP groups. This increase in the experimental groups was considered statistically different compared to the Control group (Figure 2B, EE p:0.0212(*); DRSP p<0.0001(****); EE+DRSP p<0.0001(****), respectively).

The increase in Chop expression in kidney tubule cells in the EE, DRSP, and EE+DRSP groups was statistically different compared to the control group (Figure 3B, p:0.0023(**); p:0.0002(***); p<0.0001(****), respectively). Chop nuclear stained and were significantly increased in the experimental kidneys (p<0.05), paralleled with their enhanced protein expression.

In conclusion, these findings suggest that ERS is induced and maintained in renal tubule cells of mice receiving EE, DRSP, and both.

DISCUSSION

The kidney is a vital organ for the organism to perform several fundamental functions, such as detoxification and discharge of drugs and toxic metabolites. The waste products formed from metabolism in the cells and given to the blood are removed from the blood by the kidneys (11). Also, metabolites of DRSP and EE are excreted in the urine and feces. This study was designed to find out and compare the changes induced in the kidneys of female mice after treatment with EE, DRSP, and combined (progesterone and estrogen) oral contraceptive pills.

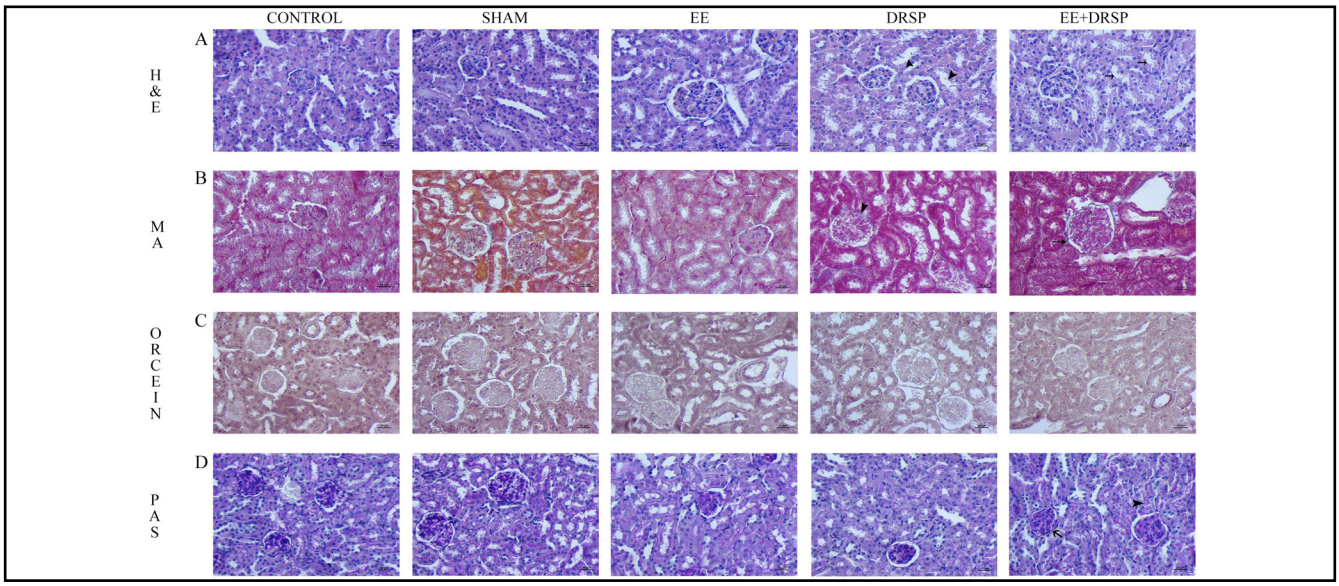

Figure 1. Histological structure and collagen, elastic fiber, and glycogen changes of kidney tissue in Control, Sham, EE, DRSP, and EE+DRSP groups. Scale bars show 40 μm and are apply to all panels. **(A)** Representative histology images of H&E staining. Section of kidney from the Control, SHAM, and EE groups showing normal tubules and normal glomeruli. Section of kidney from the DRSP (arrowheads) and EE+DRSP (arrows) groups significant loss of microvilli in proximal tubule epithelial cells **(B)** MA staining shows increased collagen fiber density around the central vein in the kidney of DRSP (arrowhead) and EE+DRSP (arrow) groups compared to the control. **(C)** Orcein staining of the kidney. Elastic fiber density no was observed to be different in all groups. **(D)** PAS staining in the kidney. Glycogen accumulation in the proximal tubules of the cortex (arrowhead) and thickening in the parietal layer of Bowman's capsule (arrow) of the EE+DRSP group.

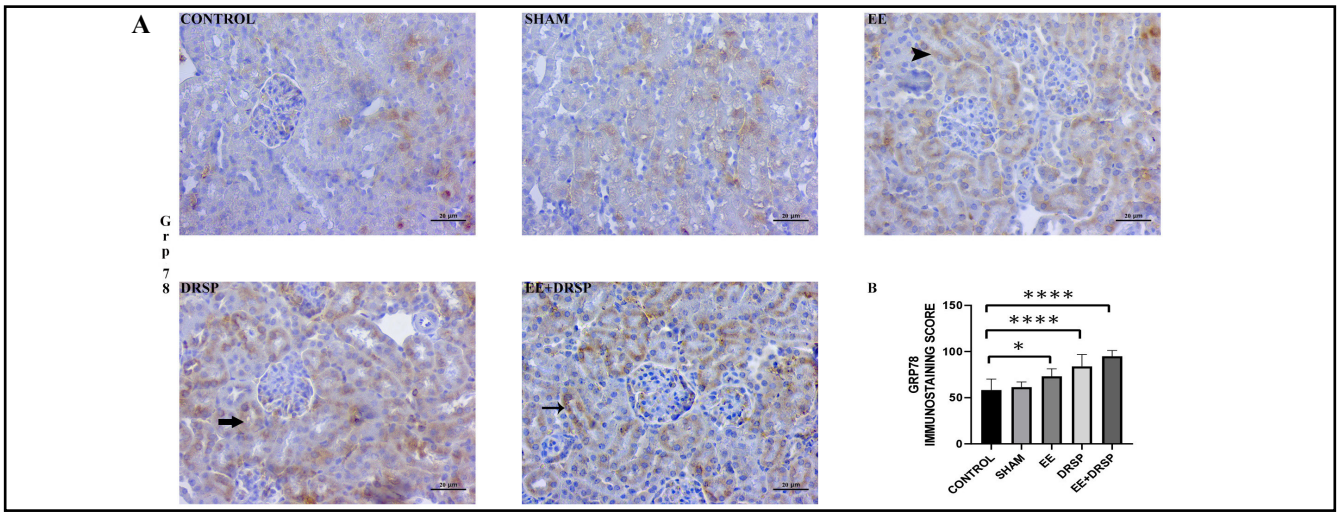

Figure 2. A. Immunohistochemical staining of ERS markers in the kidney. Representative photomicrographs showing Grp78 staining in Control, Sham, EE, DRSP, and EE+DRSP groups. Compared to the Control mice, EE (arrowhead), DRSP (thick arrow), and EE+DRSP (thin arrow) groups displayed increasingly positive immunoreactivity cells. **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. $p<0.0001$(****). Statistically significant increase in Grp78 protein expression level in EE (p:0.0212(*)), DRSP ($p<0.0001$(****)) and EE+DRSP ($p<0.0001$(****)) groups compared to the control group.

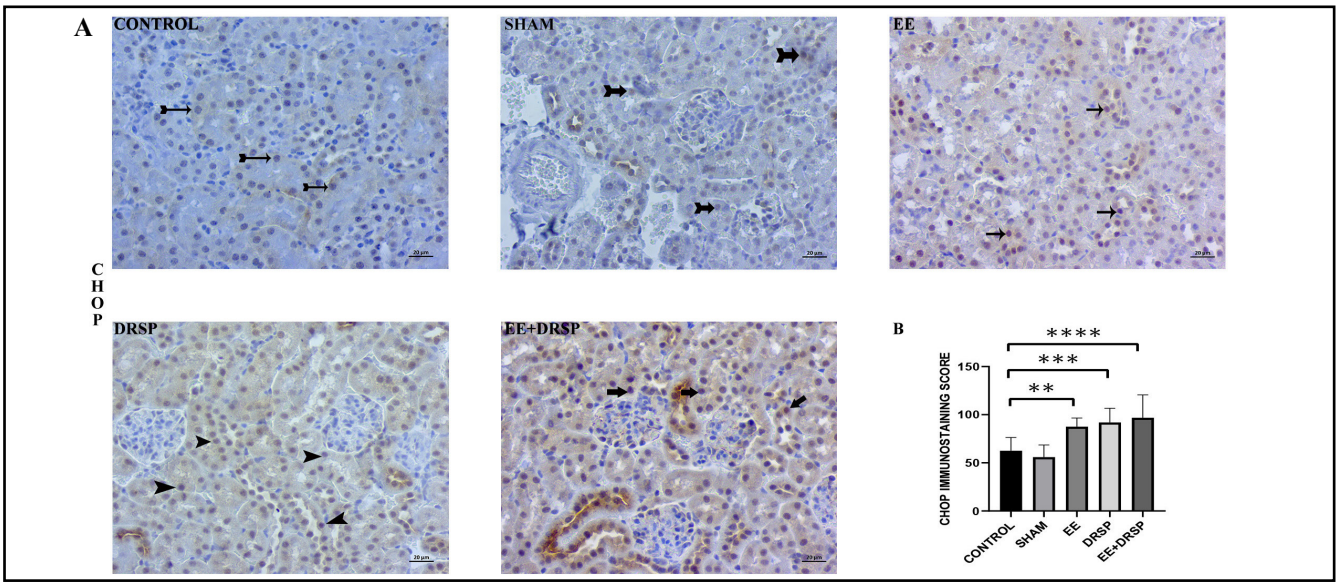

Figure 3. A. Immunohistochemical staining of ERS markers in the kidney. Representative photomicrographs show Chop staining in Control, Sham, EE, DRSP, and EE+DRSP groups. EE, DRSP, and EE+DRSP groups displayed increasingly positive immunoreactivity cells compared to the Control mice (arrows). **B.** 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 kidney tissue of EE (p:0.0023(**)), DRSP (p:0.0002(***)), and EE+DRSP (p<0.0001(****)) groups increased statistically significantly compared to the control group.

DRSP counteracts estrogen-induced stimulation of the renin-angiotensin-aldosterone system and binds to aldosterone receptors in the kidney, blocking the effects of aldosterone and has demonstrated strong antimineralocorticoid activity (17). In one study, Schürmann and colleagues reported no change in serum potassium level when comparing women with mild to moderate renal impairment to healthy controls who received 3 mg of DRSP daily for 14 days (18). While another study showed significant increase of creatinine level in women who used oral contraceptive in compared with women do not used oral contraceptive, each woman should analyze parameters that affect the kidney function before using these contraceptives (19). A study conducted on Wistar rats suggests that the use of oral contraceptives may alter the functionality of the kidney, thus leading to kidney deterioration (20).

As DRSP is a spironolactone analog with anti-mineralocorticoid activity, it can potentially induce hyperkalemia in high-risk patients with renal insufficiency. After 14 days of oral DRSP 3 mg daily in women with moderate renal impairment, mean serum DRSP levels were 37% higher than in women with normal renal function. Therefore, DRSP exposure is slightly increased in women with renal impairment; DRSP/EE 3 mg/20µg (24/4) is contraindicated in both groups (21). The study has shown that mini-pills (progesterone only) have less pronounced histological effects on the rabbits' kidneys compared to combined pills (estrogen and progesterone) (22). These results can be supported by Sitruk-Ware et al., who stated that progestin-only contraceptives are much safer than combined pills (23). In contrast, a study by Taneepanichskul et al. (24) reported that oral contraception (OC) containing DRSP was well tolerated and did not affect kidney function in women. In a further study, Al-Jomard and Al-Youzbaki (25) noticed no significant differences in the use of COC in renal function tests in women aged 19-35 years. Our study showed that increased Grp78 and Chop apoptotic markers in mouse kidneys induced ER-dependent death pathway activity give rising to apoptosis in kidneys in the use of EE, DRSP, and EE+DRSP.

Abdel Kader et al. (22) demonstrated that using combined birth control pills caused marked changes in the form of damaged rabbit's renal tubules with cell swelling, loss of the brush border, and enlarged glomeruli with hypercellularity. In addition, a statistically different increase in peritubular, peri, and intraglomerular collagen content was noticed. In parallel with these findings, Al-Ani et al. (26) observed an increase in the cellularity of the renal corpuscles of OC and

attributed this to mesangial cell proliferation. Our study reported that significant loss of microvilli in proximal tubule epithelial cells and collagen fibers increased in the parietal layer of Bowman's capsule of the kidney tissues of the ethinyl estradiol+drospirenone applied group. In parallel with our findings, PAS staining revealed thickening of tubular basement membranes in rabbits treated with the combined pill (22). Karem et al. in their biochemical analysis as a result of Yasmin administered to mice, they recorded a significant increase in the ranges of hepatic variables (AST, ALT, ALP) and kidney (creatinine) in the blood serum of all mice treated with Yasmin compared to the control group. As a result, they reported that the combined oral contraceptive tablet (Yasmin) has the potential to impair liver and kidney function and may lead to liver damage and kidney failure (27).

ERS stimulates adaptive UPR to maintain ER homeostasis and proapoptotic UPR to eliminate cells under prolonged stress, which modulates the ERS state to protect the kidney against pathogenic environments. Studies have showed a relationship between the UPR pathway and glomerular and tubular cell damage in several kidney diseases (13). Moreover, many recent studies have reported that ERS is associated with many metabolic diseases, including diabetes, chronic heart failure (28), diabetic kidney disease (29), and renal fibrosis (30). However, there has not been enough research on the kidney histological structure and immunohistochemical studies of combined oral contraceptives. More comprehensive studies are needed for the results of this effect of the long-term use of COCs on ERS.

Our study showed that increased Grp78 and Chop apoptotic markers in mouse kidneys induced ER-dependent death pathway activity leading to apoptosis in kidneys using EE, DRSP, and EE+DRSP. UPR modulators may protect kidney cells against functional dysregulation caused by COC use.

Conflict of Interest

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

Ethics Committee Approval

Permission was obtained from The Institutional Animal Ethical Committee of Akdeniz University (Antalya, Türkiye) approved the study. (Authorization reference number: 2022.01.009).

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Author's Contributions

A.C. and E.K. performed the Combined Oral Contraceptives Method on mice.

S.T. collected samples, took sections from paraffin blocks, did histological and immunohistochemical staining and statistical analysis, and wrote the manuscript.

E.K. created the project, optimizing experiments and data interpretation, and assisted to wrote the manuscript.

N.D. was the thesis advisor of E.K. and A.C. Moreover, his vast knowledge was consulted when performing the Combined Oral Contraceptives Method on mice. N.D. died on 21.12.2022.

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