Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ

Cukurova Med J 2023;48(4):1248-1257
DOI: 10.17826/cumj.1344952

RESEARCH

Comparison of effects of curcumin and beta-carotene on ovarian damage caused by cisplatin

Kurkumin ve beta-karotenin sisplatinin neden olduğu over hasarı üzerindeki etkilerinin karşılaştırılması

Ayşe Ceyhan[1,2], Münevver Baran[3], Pınar Alişan Suna[1], Özge Cengiz[1], Arzu Yay[1,4]

[1]Erciyes University, Faculty of Medicine, Department of Histology and Embryology, Kayseri, Turkey
[2]Tokat Gaziosmanpasa University, Tokat Health Services Vocational School/Medical Services and Techniques Department, Tokat, Turkey
[3]Erciyes University, Faculty of Pharmacy, Department of Basic Sciences, Kayseri, Turkey
[4] Erciyes University, Genome and Stem Cell Center (GENKOK), Kayseri, Turkey.

Abstract

Purpose: We aimed the potential protective effects of curcumin (CUR) and beta-carotene (BC) against cisplatin (CIS)-induced ovarian damage using histological and immunohistochemical methods.

Materials and Methods: We used 56 female Wistar albino rats, divided randomly into seven groups. Control rats did not receive any treatment; the sham group was administered 1 ml/kg sesame oil by gavage; the CIS group 5 mg/kg CIS; the CUR group 200 mg/kg CUR; the BC group 100 mg/kg BC; the CUR + CIS group, CIS after administration of CUR; the BC + CIS group was pretreated with BC, then administered CIS. The ovaries of all groups were excised five days after the last application. We assessed histopathology and counted and classified follicles. Expression of anti-Mullerian hormone (AMH) and nuclear factor-kappa B (NF-κB) was detected immunohistochemically. Apoptosis was evaluated using the TUNEL method.

Results: CUR and BC are protective against decreased numbers of primordial, primary, preantral, secondary, and tertiary follicles caused by CIS. Both Cur and BC reduced ovarian NF-κB levels in comparison to the control group, and AMH immunoreactivity was almost identical for the control and CUR + CIS groups. Apoptotic cell counts indicated that CUR exerts a stronger anti-apoptotic effect than BC.

Conclusion: CUR has a protective effect against ovarian damage brought on by CIS and greater antioxidant and anti-inflammatory properties than BC.

Keywords:. Anti-Mullerian hormone, apoptosis, beta-carotene, cisplatin, curcumin, NF-κB

Öz

Amaç: Cisplatin (CIS) kaynaklı over hasarına karşı kurkumin (CUR) ve beta-karotenin (BC) potansiyel koruyucu etkilerinin histolojik ve immünohistokimyasal yöntemlerle araştırılması amaçlanmıştır.

Gereç ve Yöntem: Çalışmada, rastgele yedi gruba ayrılan 56 dişi Wistar albino sıçan kullanıldı. Kontrol sıçanları herhangi bir tedavi görmedi; sham grubuna gavajla 1 ml/kg susam yağı verildi; CIS grubuna 5 mg/kg CIS; CUR grubuna 200 mg/kg CUR; BC grubuna 200 mg/kg BC; CUR + CIS grubuna, CUR uygulamasından sonra CIS uygulandı; BC + CIS grubu, BC ile ön işleme tabi tutuldu, ardından CIS uygulandı. Tüm grupların overleri son uygulamadan beş gün sonra eksize edildi. Elde edilen dokularda, histopatolojik değerlendirme yapıldı ve folikül rezervi hesaplandı. Anti-Mullerian hormon (AMH) ve nükleer faktör-kappa B (NF-κB) ekspresyonu immünohistokimyasal olarak saptandı. Apoptoz ise, TUNEL yöntemi kullanılarak değerlendirildi.

Bulgular: CUR ve BC'nin, CIS'in neden olduğu azalan primordial, primer, preantral, sekonder ve tersiyer folikül sayılarına karşı koruyucu olduğu gözlendi. AMH immünoreaktivitesi, kontrol ve CUR + CIS grupları için hemen hemen aynıydı. CUR ve BC gruplarında kontrollere kıyasla over NF-κB ekspresyon seviyelerini düşürdü. TUNEL pozitif apoptotik hücre sayıları, CUR'nin BC'den daha güçlü bir anti-apoptotik etki gösterdiğini gösterdi.

Sonuç: CIS'nin neden olduğu over hasarına karşı CUR'nin BC'den daha fazla antioksidan ve antiinflamatuar etkiye sahip olduğunu gösterilmiştir.

Anahtar kelimeler: Anti-Mullerian hormone, apoptosis, beta-karoten, cisplatin, kurkumin, NF-κB

Address for Correspondence: Arzu Yay, Department of Histology and Embryology, Medicine Faculty, University of Erciyes, Kayseri, Turkey, and Genome and Stem Cell Center (GENKOK), Erciyes University, Kayseri, Turkey.
E-mail: arzu.yay38@gmail.com
Received: 25.08.2023 Accepted: 16.11.2023

INTRODUCTION

Chemotherapy induces ovarian failure and infertility in female cancer patients by depleting the primordial follicle supply in their ovaries[1]. Menopause and the loss of female fertility are caused by the ovary primordial follicle complement[2]. Cisplatin (cis-diaminodichloroplatin II, CIS) is an effective antineoplastic drug that is usually used to treat cancers including head and neck, bladder, testis, cervix, esophagus, ovary, estrogenic sarcoma, and neuroblastoma[3].

It affects cancer cells by creating adducts and causing DNA damage, which stops the cell cycle and leads to apoptosis[4]. Furthermore, CIS can internalize and disrupt the homeostasis of various organelles, including the nucleus, mitochondria, lysosomes, and endoplasmic reticulum[5]. However, anticancer drugs may have harmful cytotoxic effects on healthy tissues and cells, such as the reproductive system. Chemotherapy may reduce quality of life by affecting sexual hormones, organ structure, function, and fertility, even leading to fertility loss[6]. Therefore, many female patients will have undesirable gonadotoxic side effects from cancer treatments, so fertility preservation is crucial, particularly for younger patients. The use of antioxidants together with chemotherapeutics has become the standard alternative treatment method for decreasing tissue damage following chemotherapy.

Medical plants are a source of therapeutic agents for human diseases. It is widely considered that natural drugs are safer than synthetic drugs[7]. Curcumin (CUR) and beta-carotene (BC) are strong antioxidants[8,9]. CUR is a natural diarylheptanoid pigment and the most active component of turmeric. In addition to its antioxidant properties, CUR is an antitumor, anti-arthritic, anti-amyloidal, anti-ischemic, antimicrobial, and anti-inflammatory agent[10]. CUR prevents lipid oxidation and oxidative DNA damage; it reduces the release of arachidonic acid formed by inhibition of oxidative lipoxygenase and cyclo-oxygenase enzymes[11]. BC is a lipid-soluble carotenoid vitamin precursor (pro-vitamin); its active form is vitamin A, which is found in many fruits and vegetables[12]. BC provides protection against oxidative damage. It protects against ischemic-reperfusion damage in the liver, kidney, and heart[13-14].

Do the CUR and BC have protective effects against ovarian damage caused by CIS? In light of the literature data summarized above, the hypothesis of the study is that the antioxidant effect of CUR and BC is expected to cause ovarian damage caused by cisplatin. Therefore, in the current study, we evaluated the ovarian damage caused by CIS administration as well as the potential preventive benefits of CUR and BC using histochemical techniques.

MATERIALS AND METHODS

Animals

A total of 56 Wistar albino female rats, 8-10 weeks old, were purchased from the Erciyes University Experimental Research Center in accordance with the ethical rules of standard experimental practices. Our study was approved by the Erciyes University Local Ethical Committee of Animal Experiments (Ethics Committee No. 16/124 dated 12.10.2016). The care and housing of the experimental animals were carried out at the Experimental and Clinic Research Center at Erciyes University. The study was performed in the Histology and Embryology Laboratory, Faculty of Medicine, Erciyes University. The experimental procedure was followed by researchers, who obtained a certificate of experimental animal use.

Treatments and experimental design

The CIS group was administered a single 5 mg/kg/week dose of CIS (Sigma-Aldrich, Saint Louis, MO) dissolved in normal saline by intraperitoneal injection (i.p.). The CUR group was administered 200 mg/kg/week of CUR orally (Sigma-Aldrich, Saint Louis, MO)[15]. The BC group was administered 100 mg/kg/week of BC (Sigma-Aldrich) dissolved in sesame oil prior to CIS administration orally. The CIS + BC group orally received BC for 30 minutes before receiving CIS, and the CIS + CUR group orally received CUR for 30 minutes before receiving CIS. One week following the first application, the second application was performed[16].

The rats were divided randomly into seven groups of eight. Control rats were untreated. Sesame oil (1 mL/kg) was given to the sham group. The CIS group was administered 5 mg/kg of CIS i.p. The CUR group was administered 200 mg/kg of CUR by gavage[17]. The BC group was administered 100 mg/kg

BC in sesame oil by gavage[18]. The CUR + CIS group was administered CIS 30 minutes after CUR. The BC + CIS group was administered CIS 30 minutes after receiving BC. The same time of day was used for all of the treatments. During the second administration, one week after the first, the experimental groups received the same i.p. and/or gavage procedures as in the first. Rats were anesthetized by i.p. injections of 10 mg/kg xylazine (Rompun; Bayer, Leverkusen, Germany) and 50 mg/kg ketamine (Ketalar Flacon; Pfizer, New York) five days after the second treatment (day 12). Ovaries were dissected and excised, then washed with saline. Ovaries were fixed with 10% formalin for histopathological examination.

Histopathology

The tissues were dehydrated with ascending alcohols after being fixed in formalin, cleaned with xylene, and then embedded in parafin. Serial sections that were 5 µm thick were cut and attached to slides. For the purpose of determining the histopathology, sections were deparaffinized with xylene, rehydrated, and stained with hematoxylin and eosin (H&E) or Masson trichrome. Using an Olympus BX51 microscope (Tokyo, Japan) with a DP-71 camera, sections were analyzed and captured.

Classification and counting of follicles

A histologist who was not familiar with the process counted and labeled folliculles on sections stained with H&E. As previously mentioned, follicles were categorized as primordial, preantral, antral, or atretic[19]. One of twelve serial ovarian sections from each subject was examined by light microscopy to determine the follicular reserve. Primordial, primary, preantral, secondary, and tertiary follicles, whose nuclei and nucleoli could be observed clearly, were counted. Granulosa cell shape and number of cell layers were considered for the morphological classification of follicles. Healthy follicles exhibited an intact basal lamina, an oocyte, an intact germinal vesicle, and an undamaged nucleolus. Damaged follicles were identified by lost granulosa cells and pyknotic nuclei.

Immunohistochemistry

Ovarian tissue blocks were cut into five micron-thick cross-sections and subsequently deparaffinized, rehydrated and washed three times with phosphate-buffered saline (PBS) at room temperature. The manufacturer's instructions were followed while performing an avidin-biotin-peroxidase technique for immunostaining. The following procedures were carried out using an anti-polyvalent HRP kit from Thermo Scientific, USA: Anti-Mullerian hormone (AMH) (sc 1667529; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-NF-κB antibody (sc-8008; Santa Cruz Biotechnology) were incubated overnight at 4 °C. As a check, primary antibodies were not used. The slices were then treated with a biotinylated secondary antibody (Thermo Scientific, USA) following PBS washing. PBS was used to clean the sections before peroxidase-conjugated streptavidin was applied for 10–15 minutes at room temperature. Finally, 4',6-diamidino-2-phenylindole (Thermo Scientific, USA) was used for 2 minutes to visualize the immunostaining. It was counterstained with Mayer's hematoxylin. Using a light microscope (BX51; Olympus Tokyo, Japan), immunostaining was seen. Using The intensity of NF-kB immunostaining was evaluated from ten microscopic fields per animal (a total of 100 microscopic fields) using Image J software (ImageJ, Bethesda, MD) at an original magnification of 400 x. In sections of each experimental group, we selected primordial, primary, preantral, and secondary follicles at the same magnification. Using Image J software, the level of AMH immunoreactivity for each follicle was estimated and recorded.

Terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining

Using the TUNEL method, apoptotic cells were detected. We followed the manufacturer's recommendations when using a TUNEL apoptosis detection kit (ApopTag fluorescein in situ apoptosis detection kit; Millipore, Berlin, Germany). Following deparaffinization, rehydration, and two 5-minute PBS washes, sections were incubated with digoxigenin-dUTP for an hour at 37 °C in the presence of terminal deoxynucleotidyl transferase (TdT). The TUNEL kit (in situ TdT-mediated X-dUTP-nicked labeling (TUNEL) reaction using the ApopTag® Fluorescein In Situ Apoptosis Detection Kit, EMD Millipore, Darmstadt, Germany) was used to identify TUNEL-positive cells using an anti-digoxigenin fluorescence unconjugated antibody. At a magnification of 400, at least ten random fields in each area were tallied. Using an Olympus BX51 microscope, all slides have been examined and

captured on camera. Using ImageJ software, the quantity of apoptotic cells was calculated by counting TUNEL-positive cells[13].

Statistical analysis

Software version 3.2.2 of R (www.r-project.org) was used to analyze the data. The Shapiro-Wilk test, q-q graphics, and a histogram were used to assess the data's conformity to the normal distribution. The Levena test was used to evaluate the homogeneity of the variance. For intergroup comparisons, one-way variance analysis (number of follicles and immunreactivity intensities (AMH and NF-ϰB) and the Kruskal-Wallis test (TUNEL staining) were used. Determining the number of subjects to be included in the study yielded a 98% confidence interval and 97% test power. For multiple comparisons, the Dunn-Bonferroni, Tukey HSD, and Tamhane tests were applied. The level of significance was defined as $p \leq 0.05$.

RESULTS

Histopathology

The control ovaries showed up as normal in H&E-stained sections, and they had capillaries in the medulla and primordial, primary, pre-antral, and antral follicles in the cortex, as shown in Figure 1. In the sesame oil group, the medulla was primarily made up of loose connective tissue, while the cortex included many follicles of varying sizes and developmental stages. The CIS-treated group exhibited decreased follicle maturation, vascular congestion, stromal hyperplasia, and hemorrhage compared to the control group. The CIS group exhibited numerous degenerated follicles and significant edema; oocytes appeared degenerated compared to the control group. The ovarian sections from animals treated with CUR exhibited normal follicles at various phases in the cortex. Preantral stage follicles in the CUR + CIS and BC + CIS groups were also found to have normal primordial follicles, in contrast to the CIS group, where they were in the early stages of atresia. We found that the protective effect of BC was less than that of CUR. Masson's trichrome staining revealed no significant changes in collagen in rat ovaries following exposure to CIS (Figure 1).

Number of follicles

To understand how ovarian follicle differentiation and development were affected by CUR or BC treatment, various ovarian follicle classifications, including primordial, primary, secondary, antral, and atretic were identified. Figure 1 shows the histological features of follicles at each stage. We counted the typical number of follicles at each stage (Table 1).

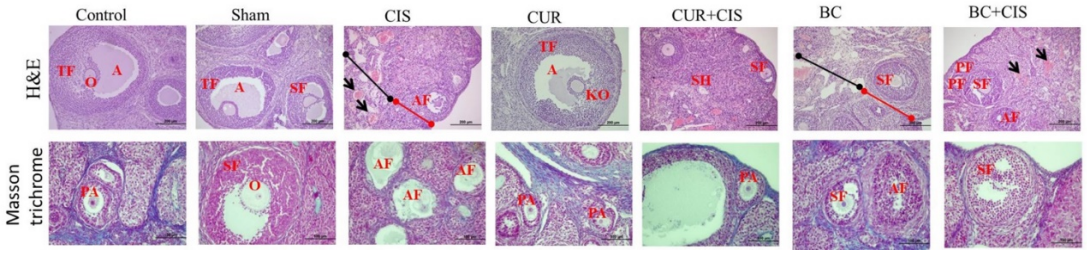

Figure 1. The control group appeared normal. Red line, cortex; PF, primary follicle. Sham group. PF, normal primary follicle; SF, secondary follicle; A, antrum; O, oocyte; CL, corpus luteum. The CIS group exhibited follicular degeneration and hemorrhage. AF, atretic follicle; black arrow, hemorrhage; *, edema. CUR group. PF, primary follicle; TF, tertiary follicle; O, oocyte; SH, stromal cells. CUR + CIS group PF, primary follicle; SF, secondary follicle; CL, corpus luteum. BC group. PF, primary follicle; O, oocyte. BC+CIS group AF, atretic follicle; SF, secondary follicle. H&E staining. Masson trichrome staining: primary follicle (PF) and secondary follicle (SF) in the ovarian section of the control group Secondary follicle (SF) and oocyte (O) in the ovary section of the Sham group. Atretic follicles (AF) in the ovarian section of the CIS group CUR group belonging to the secondary follicle (SF) Preantral follicle (PA), primary follicle (PF), and secondary follicle (SF) in the ovarian section of the CUR+CIS group; in the ovarian section of the BC group, secondary (SF) and atretic follicles (AF). BC+CIS group; secondary follicle (SF); hemorrhage (black arrow) (x20 magnification).

The number of primordial, secondary, tertiary, and atretic follicles differed significantly amongst the groups, according to statistics. In comparison to the control group, there were fewer primordial follicles in the CIS group. In terms of primary, preantral, or secondary follicles, there was no noticeable distinction between the CIS group and other groups. There were significantly fewer mature and atretic follicles in the CIS-treated group compared to the control group, but there were no discernible changes between the CIS + CUR and BC + CUR groups. In comparison to the CIS group, rats in the CIS + CUR group had more total ovarian follicles and considerably fewer atretic follicles ($p < 0.05$). The control and CUR + CIS groups contained the most primary, preantral, and secondary follicles. The number of follicles between the control and CIS+CUR groups showed no discernible variation. We discovered that, compared to BC, CUR showed a higher protective impact on follicular reserve.

Table 1. Total primordial, primary, preantral, secondary, tertiary and atretic follicles.

Follicles	Control	Sham	CIS	CUR	CUR + CIS	BC	BC + CIS	*p*
Primordial	136.33 ± 28.41[a]	119.33 ± 25.54[a,b]	87.00 ± 12.23[b]	116.50 ± 15.87[a,b]	123,00 ± 37.06[a,b]	121.83 ± 29.02[a,b]	92.33 ± 13.97[a, b]	0.016
Primary	59.00 ± 6.03	50.83 ± 8.23	42.00 ± 12.59	47.50 ± 6.83	54.17 ± 10.98	47.83 ± 19.18	42.50 ± 20.83	0.293
Preantral	28.33 ± 4.72	24.83 ± 11.03	20.50 ± 4.64	24.00 ± 4.18	29.00 ± 6.81	24.50 ± 4.04	26.33 ± 7.37	0.351
Secondary	22.68 ± 6.05	20.17 ± 7.60	12.33 ± 2.66	21.33 ± 7.45	21.67 ± 7.20	19.17 ± 5.78	17.67 ± 3.56	0.091
Tertiary	7.67 ± 4.46	10.33 ± 2.80	1.83 ± 0.98[b]	8.17 ± 2.93	9.17 ± 1.94	8.33 ± 2.34	7.33 ± 2.25	< 0.001
Atretic	32.17 ± 10.32[a]	52.67 ± 13.34[a]	108.50 ± 15.21[b]	60.83 ± 21.42[a]	80.83 ± 30.04[a,b]	73.67 ± 18.15[a,b]	94.83 ± 22.07[b]	< 0.001

The data are expressed as the mean ± standard deviation for seven rats in each group. The same letters in the same row show similarity between groups, and different letters indicate differences between groups. p-value of <0.05 was used for significance. Control: untreated group; Sham: the group that received only sesame oil; CIS: cisplatin group; CUR: curcumin group; CUR+CIS: combined therapies with CIS and CUR; BC: beta-carotene group; BC+CIS: combined therapies with CIS and BC.

Immunohistochemistry

The AMH staining of the specimens is shown in Figure 2. AMH expression was observed in primary, preantral, secondary, and tertiary follicles. AMH-positive cells have been identified in the cytoplasm of granulosa cells at the early follicle stage and in every granulosa cell of the primary and small antral follicles in the control group, but there was no AMH immunostaining in the larger antral follicles. Similarly, no AMH expression was found in primordial follicles, germ cells, or thecal cells in the control group. The intensity of AMH immunostaining in follicles of the CIS, BC, and BC + CIS groups was significantly less than that of the control group. We found no significant difference in the intensity of AMH immunostaining in tertiary follicles in any group ($p < 0.05$) (Table 2). CUR increased AMH expression more than BC. Therefore, CUR exhibited a stronger protective effect. Immunostaining of NF-κβ for the CUR and BC groups revealed mild immunostaining for NF-κβ. The ovarian tissue in the CIS group exhibited stromal cells with strong NF-κβ cytoplasmic immunostaining. Sections from the CIS group displayed higher NF-κβ immunoreactivity in the theca cells and ovarian stromal cells ($p<0.05$) in comparison to the control group (Table 3). In comparison to the ovaries exposed to CIS, the CIS + CUR group's stromal cells showed moderate NF-κβ immunoreactivity, indicating a significant down-regulation of NF-κβ expression. Sections of CIS ovaries treated with CUR showed lower levels of NF-κβ immunoreactivity than ovaries in the CIS group. Both CUR and BC decreased the increased immunoreactivity of CIS-induced NF-κβ. Images of ovary tissues are shown in Figure 2.

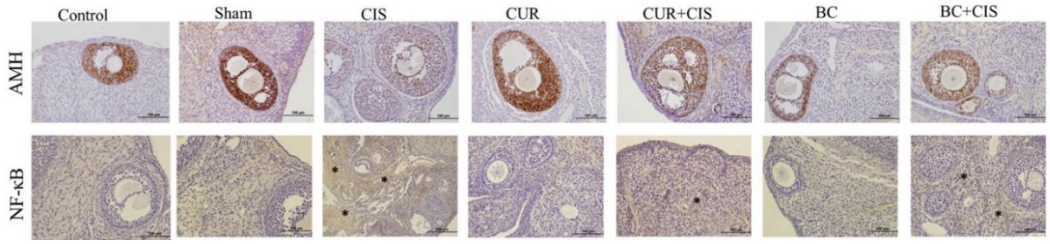

Figure 2. Immunostaining images of anti-AMH in the ovaries, including all study groups Immunohistochemical results of AMH in follicles at varied stages appear as a deep brown cytoplasmic reaction in granulosa cells of developing follicles in the control group, while little reaction is apparent in the CIS group. IHC staining counterstained with hematoxylin. x 400. AMH immunostaining. No significant immunoreactivity was observed in the control and sham groups. Intense immunoreactivity was observed only in the CIS group. Both CUR and BC reduced the increased immunoreactivity. NF-κβ immunostaining counterstained with hematoxylin.

Table 2. AMH immunoreactivity in primordial, primary, preantral, secondary, tertiary follicles.

Follicles	Control	Sham	CIS	CUR	CUR + CIS	BC	BC + CIS	*p*
Primordial	110.32 ± 13.01^{a}	102.50 ± 9.00^{b}	102.50 ± 9.3^{b}	107.96 ± 12.03^{a}	103.9 ± 11.56^{a,b}	98.02 ± 9.16^{b}	98.5 ± 7.60^{b}	< 0.001
Primary	107.72 ± 17.54^{a}	101.61 ± 10.61^{a}	90.44 ± 10.37^{b}	103.69 ± 14.93^{a}	99.44 ± 8.44^{a}	97.87 ± 10.27^{a,b}	94.8 ± 13.26^{a,b}	< 0.001
Preantral	90.68 ± 5.53^{a}	92.01 ± 6.93^{a}	74.42 ± 8.69^{b}	88.64 ± 6.35^{a,b}	88.06 ± 6.50^{a,b}	82.44 ± 8.29^{a,b}	80.9 ± 6.58^{b}	0.001
Secondary	79.00 ± 13.14	70.53 ± 11.16	62.67 ± 9.43	67.56 ± 4.90	67.26 ± 5.66	72.21 ± 5.50	70.0 ± 5.53	0.302

Data are expressed as the mean ± standard deviation. The same letters in the same row show similarity between groups, and different letters indicate differences between groups. Control: untreated group; Sham: the group that received only sesame oil; CIS: cisplatin group; CUR: curcumin group; CUR+CIS: combined therapies with CIS and CUR; BC: beta-carotene group; BC+CIS: combined therapies with CIS and BC.

Table 3. Immunostaining intensity for NF-kB in experimental groups.

	Control	Sham	CIS	CUR	CUR + CIS	BC	BC + CIS	*p*
NF-κB	81.00 ± 4,07^{a}	83.72 ± 4.73^{a}	92.28 ± 9.74^{b}	83.65 ± 4.70^{a}	83.87 ± 5.92^{a}	82.02 ± 7.26 ^{a}	82.36 ± 4.45^{a}	< 0.001

Data are expressed as the mean ± standard deviation. The same letters in the same row show similarity between groups, and different letters indicate differences between groups. Control: untreated group; Sham: the group that received only sesame oil; CIS: cisplatin group; CUR: curcumin group; CUR+CIS: combined therapies with CIS and CUR; BC: beta-carotene group; BC+CIS: combined therapies with CIS and BC. NF-κB: nuclear factor-kappa B

TUNEL staining

TUNEL-positive cells were observed in both the medulla and cortex of the ovaries in all experimental groups. TUNEL-stained cells were detected in the ovarian granulosa and theca cells, as well as interstitial cells. The CIS group markedly increased the number of TUNEL-positive apoptotic cells (Figure 3) compared to all other experimental groups ($p < 0.001$) (Table 4). The number of TUNEL-positive apoptotic cells in the CUR + CIS group was significantly lower than the CIS group ($p < 0.001$) and was similar to the control group.

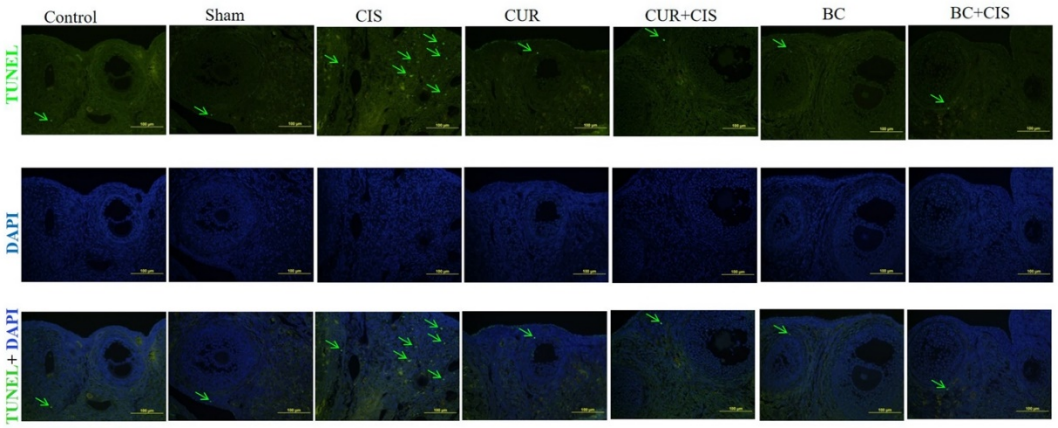

Figure 3. TUNEL-positive cells reflect green immunofluorescence. Positive apoptotic cells were counterstained with DAPI nuclear staining. TUNEL-positive apoptotic cells (green arrow) were decreased on section profiles of the Control and Sham groups. TUNEL-positive apoptotic cells showed CUR and BC groups. There were many apoptotic cells in the ovary sections of group CIS. There were only a few TUNEL-positive cells in the ovaries of groups CUR+CIS and BC+CIS (TUNEL, DAPI, and TUNEL+DAPI). Many apoptotic cells appear in the CIS group. Only a few TUNEL-positive cells appear in the CUR + CIS and BC + CIS groups. Tunel staining is counterstained with DAPI nuclear staining.

Table 4. Quantification of apoptotic cells in ovarian sections, identified by TUNEL assay.

	Control	Sham	CIS	CUR	CUR + CIS	BC	BC + CIS	*p*
TUNEL-positive cell	0.0 (0.0-1.0)^{a}	0.0 (0.0-1.0)^{a}	5.5 (3.0-7.75)^{c}	1.00 (0.0-2.0)^{a}	1.0 (0.0-2.0) ^{a}	1.0 (0.0-2.0) ^{a}	1.0 (1.0-2.0)^{b}	< 0.001

The data are expressed as the median (quarter 1–quarter 3). The same letters in the same row show similarity between groups, and different letters indicate differences between groups. Control: untreated group; Sham: the group that received only sesame oil; CIS: cisplatin group; CUR: curcumin group; CUR+CIS: combined therapies with CIS and CUR; BC: beta-carotene group; BC+CIS: combined therapies with CIS and BC. TUNEL: Terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP nick end labeling

DISCUSSION

CIS is an antineoplastic drug that impairs fertility[20]. It is an effective chemotherapeutic agent for treating sarcomas, lymphomas, and testicular, ovarian, breast, lung, and bladder cancers[21]. CIS treatment is accompanied by toxic side effects, including reproductive toxicity and an increased frequency of germ cell apoptosis[22]. In this study, we observed that CIS exerts detrimental effects on both growing and primordial ovarian follicles, which reduces ovarian follicle reserve.

CUR and BC are antioxidants derived from plants that exhibit tissue protective potential[8,9]. CUR is protective against tissue injury in the liver[23], heart, nervous system[24] and gonads[25]. CUR exhibits anti-inflammatory, antioxidant, and anticancer properties[24,26]. CUR is protective against oxidation due to ischemic-reperfusion injury[17]. BC is a precursor of vitamin A; it is an antioxidant that contributes to the color of fruits and vegetables and participates in photosynthesis. BC helps counter the effects of oxidation[13]. We investigated the mechanisms by which CUR and BC attenuate CIS-induced ovarian toxicity using H&E and Masson trichrome staining, immunostaining of AMH and NF-κβ, and TUNEL staining methods. We found severe oxidative damage in the CIS group compared to the control group. We observed significantly decreased vascular congestion, stromal hyperplasia, and hemorrhage in both the CUR + CIS and CUR + BC groups compared to the CIS group.

Chemotherapy can lead to changes in sexual hormones, organ structure, function, and fertility. It can even result in fertility loss, which decreases the quality of life. Moreover, anticancer drugs may be harmful to healthy cells and tissues, including the reproductive system, by causing cytotoxicity[6]. There have been few reports concerning ovarian damage caused by chemotherapy and the effects of oxidants on ovarian follicles. CIS is detrimental to both growing and primordial ovarian follicles, which causes a permanent reduction of the ovarian follicle reserve[27,28]. As a result, the chemotherapy regimens for this drug have been restricted[29]. CUR promotes folliculogenesis and reduces apoptosis in murine ovaries[30,31]. In addition to the findings from these studies, we also found that CUR produced a stronger protective effect on the follicle reserve than BC. Therefore, this suggests that CUR might promote follicular development or maintain primordial follicles.

AMH is frequently used for investigations into ovarian damage[32] and is a marker for ovarian aging[33]. It also represents the size of the ovarian follicle pool. We discovered that CIS significantly decreased ovarian AMH expression. We identified AMH expression in granulosa cells in the control group's primary, preantral, secondary, and tertiary follicles, but not in primordial follicle cells, which are a layer of squamous granulosa cells that surround an oocyte. We found little AMH expression in late secondary follicles or in granulosa and cumulus cells of tertiary follicles. Therefore, AMH expression is a useful indicator of ovarian follicle reserve, as reported by Visser et al.[34] According to Hansen et al.[35], AMH expression is strongly connected to the quantity of antral follicles. This study clearly showed that CIS significantly reduced the AMH immunoreactivity in the granulosa cells of significant ovarian follicles. According to our research, CUR promotes more effective follicle growth and increases AMH levels in the ovaries of the CIS+CUR group compared to the CIS+BC group. The findings may thus provide some novel evidence that CUR is more effective at protecting against and treating impairments to female reproductive systems caused by CIS than BC. We localized NF-κβ in the ovaries using immunohistochemistry. The CIS treatment increases the production of NF-κβ, an indicator of oxidative stress. We found that NF-κβ immunostaining was significantly increased in the CIS group as in literature[36]; this reactivity was reduced in the BC groups. NF-κβ expression is increased in many cancers, and it is associated with the development of malignancy. CUR may be a direct scavenger of free radicals and may also modify the signaling pathways controlled by NF-κβ[37]. We found that both CUR and BC decrease the increased immunoreactivity of CIS-induced NF-κβ; both CUR and BC exhibit protective effects.

The intricate interplay between inflammation and apoptosis results in ovarian damage induced by CIS. CIS creates DNA crosslinks, which in turn cause DNA breaks, which initiate apoptosis and exhaust the follicle pool, which results in ovarian failure[38]. Chemotherapy supposedly targets cells that are actively dividing. Therefore, the appropriate targets for chemotherapy should be the proliferating somatic cells surrounding developing or mature follicles rather than mitotically inactive cells[39]. Depletion of follicles due to apoptosis stimulates primordial follicles, leading to more precocious exhaustion of the follicular pool[40]. Taskın et al.[15] reported that CIS induced apoptosis in the ovary. Our findings support the current study showing a decrease in follicle count and increase in apoptosis in the CIS group, as well as an increased prevalence of TUNEL-positive cells in primary, secondary, and tertiary follicles with cisplatin treatment. Consistent with our AMH and NF-κβ histochemical findings, when compared to the CIS group, we showed that the CUR + CIS and BC + CIS groups had considerably fewer TUNEL-positive apoptotic cells. Comparable to the control group, the CUR+CIS group had a similar number of TUNEL-positive apoptotic cells. The protective effect of BC against ischemia-reperfusion injury to ovarian tissue was described by Aksak et al.[18]. The CIS group had the highest percentage of TUNEL-positive apoptotic cells; however, as compared to the BC group, CUR dramatically reduced the number of TUNEL-positive cells, indicating a protective effect against CIS-induced ovarian apoptosis.

The histological structure of ovaries that were administered CUR or BC was preserved after CIS application. The results of follicle count analysis revealed that CIS treatment reduced ovarian reserve capacity, but CUR treatment increased the number of follicles relative to the BC+CIS group. Furthermore, CIS reduces AMH immunoreactivity in follicles while increasing the number of TUNEL-positive cells and NF-κβ immunoreactivity. Although BC did not completely prevent ovary injury resulting from CIS-induced ovarian toxicity, the extent of the ovary damage was diminished compared to CIS treatment

alone. Our findings indicate that CUR has more anti-oxidant and anti-inflammatory effects than BC on ovarian CIS damage.

The current study has certain limitations, despite resulting in valuable results. The main limitation is the requirement for additional biochemical data, particularly for parameters like total antioxidant level (TAS), total oxidant level (TOS), and GSH-Px, given that both CUR and BC are antioxidant compounds. Furthermore, the results can be even more reliable if molecular analyses are used to verify the data collected from the studies. Studies studying apoptotic markers in CIS-induced ovarian damage are very scarce in the literature, and the current study's results, which departed from those of the previous investigations, have brought attention to the need for a more comprehensive assessment of the topic. We believe that by taking into account the findings and limitations of the current study, future research can produce more convincing evidence.

Author Contributions: Concept/Design : AC, AY; Data acquisition: AC, ÖC; Data analysis and interpretation: AC, MB, AY; Drafting manuscript: AC, AY; Critical revision of manuscript: AC, MB, PAS, ÖC, AY; Final approval and accountability: AC, MB, PAS, ÖC, AY; Technical or material support: AC, ÖC; Supervision: MB, AY; Securing funding (if available): n/a.
Ethical Approval: Ethical approval was obtained from the Local Ethics Committee of Animal Experiments of Erciyes University with the decision dated 12.10.2016 and numbered 16/124-9.
Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest.
Financial Disclosure: This study was supported by grants from the Erciyes University Scientific Research Projects Unit, project no. TYL-2016-7069.

REFERENCES

1. Hansen KR, Knowlton NS, Thyer AC, Charleston JS, Soules MR, Klein NA. A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause. Hum Reprod. 2008;23:699–708.
2. Rossi V, Lispi M, Longobardi S, Mattei M, Di Rella F, Salustri A et al. LH prevents cisplatin-induced apoptosis in oocytes and preserves female fertility in mouse. Cell Death Differ. 2017;24:72–82.
3. Brown A, Kumar S, Tchounwou P. Cisplatin-based chemotherapy of human cancers. J Cancer Sci Ther. 2011;11:97–103.
4. Gouveia BB, Barberino RS, Menezes VG, Monte APO, Silva RLS, Palheta RC Jr et al. Amburana cearensis leaf extract protects against cisplatin-induced ovarian damage through regulation of p-PTEN and p-Akt proteins in mice. Iran J Basic Med Sci. 2022;25:683-9.
5. Gąsiorkiewicz BM, Koczurkiewicz-Adamczyk P, Piska K, Pękala E. Autophagy modulating agents as chemosensitizers for cisplatin therapy in cancer. Invest New Drugs. 2021;39:538-63.
6. Anderson RA, Clatot F, Demeestere I, Lambertini M, Morgan A, Nelson SM et al. Cancer survivorship: Reproductive health outcomes should be included in standard toxicity assessments. Eur J Cancer. 2021;144:310-16.
7. Pandya U, Saini MK, Jin GF, Awasthi S, Godley BF, Awasthi YC. Dietary curcumin prevents ocular toxicity of naphthalene in rats. Toxicol Lett. 2000;115:195-204.
8. Farombi EO, Ekor M. Curcumin attenuates gentamicin-induced renal oxidative damage in rats. Food Chem Toxicol. 2006;44:1443-8.
9. Menon VP, Sudheer AR. Antioxidant and anti-inflammatory properties of Curcumin. Adv Exp Med Biol. 2007;595:105–25.
10. Devasena T, Rajasekaran KN, Menon VP. Bis-1,7-(2-hydroxyphenyl)-hepta-1,6-diene-3,5-dione (a curcumin analog) ameliorates DMH-induced hepatic oxidative stress during colon carcinogenesis. Pharmacol Res. 2002;46:39-45.
11. El-Demerdash FM, Yousef MI, Kedwany FS, Baghdadi HH. Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of malerats: protective role of vitamin E and beta-carotene. Food Chem Toxicol. 2004;42:1563–71.
12. Codoner-Franch P, Muniz P, Gasco E, Domingo JV, Valls-Belles V. Effect of diet supplemented with alpha-tocopherol and beta-carotene on ATP andantioxidant levels after hepatic ischemia-reperfusion. J Clin Biochem Nutr. 2008;43:13-18.
13. Hosseini F, Naseri MK, Badavi M, Ghaffari MA, Shahbazian H, Rashidi I. Effect of beta carotene on lipid peroxidation and antioxidant status following renal ischemia/reperfusion injury in rat. Scand J Clin Lab Invest. 2010;70:259-63.
14. Taskın MI, Yay A, Adalı E, Balcıoglu E, Inceboz U. Protective effects of sildenafil citrate administration on cisplatin induced ovarian damage in rats. Gynecol Endocrinol. 2015;31:272-7.
15. Soleimani H, Abdollah A, Taheri S, Sajadi E, Shafikhani S, Schuger LA et al. The effect of combined photobiomodulation and curcumin on skin wound healing in type I diabetes in rats. J Photochem Photobiol B. 2018;181:23-30.
16. Eser A, Hizli D, Haltas H, Namuslu M, Kosus A, Kosus N et al. Effects of curcumin on ovarian ischemia-reperfusion injury in a rat model. Biomed Rep. 2015;3:807-13.
17. Karamese Aksak S, Toktay E, Unal D, Sellik J, Karamese M, Malkoc I. The protective effects of beta-carotene against ischemia/reperfusion injury in rat ovarian tissue. Acta Histochem. 2015;117:790-7.
18. Sonmezer M, Oktay K. Fertility reservation in female patients. Hum Reprod Update. 2004;10:251–66.

19. Brozovic A, Ambriović-Ristov A, Osmak M. The relationship between cisplatin-induced reactive oxygen species, glutathione, and BCL-2 and resistance to cisplatin. Crit Rev Toxicol. 2010;40:347–59.
20. Amin A, Buratovich M. New platinum and ruthenium complexes—the latest class of potential chemotherapeutic drugs-a review of recent developments in the field. Mini Rev Med Chem. 2009;9:1489-503.
21. Fung C, Vaughn DJ. Complications associated with chemotherapy in testicular cancer management. Nat Rev Urol. 2011;8:213-22.
22. Mohebbati R, Anaeigoudari A, Khazdair MR. The effects of Curcuma longa and curcumin on reproductive systems. Endocr Reg. 2017;51:220-8.
23. Aggarwal BB, Harikumar KB. Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neuro degenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. Int J Biochem Cell Biol. 2009;41:40–59.
24. Wang XN, Zhang CJ, Diao HL, Zhang Y. Protective effects of curcumin against sodium arsenite-induced ovarian oxidative injury in a mouse model. Chin Med J. 2017;130:1026-32.
25. Wilken R, Veena MS, Wang MB, Srivatsan ES. Curcumin a review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma. Mol Cancer. 2011;10:1-19.
26. Mark-Kappeler CJ, Hoyer PB, Devine PJ. Xenobiotic effects on ovarian preantral follicles. Biol Reprod. 2011;85:871–83.
27. Atli M, Engin-Ustun Y, Tokmak A, Caydere M, Hucumenoglu S, Topcuoglu C. Dose dependent effect of resveratrol in preventing cisplatin-induced ovarian damage in rats: an experimental study. Reprod Biol. 2017;17:274–80.
28. Un H, Ugan RA, Kose D, Bayir Y, Cadirci E, Selli J, Halici Z. A novel effect of aprepitant: protection for cisplatin-induced nephrotoxicity and hepatotoxicity. Eur J. Pharmacol. 2020;880:173168.
29. Voznesens'ka T, Bryzhina TM, Sukhina VS, Makohon NV, Aleksieieva IM. Effect of NF-kappaB activation inhibitor curcumin on the oogenesis and follicular cell death in immune ovarian failure in mice. Fiziol Zh. 2010;56:96–101.
30. Aktas C, Kanter M, Kocak Z. Antiapoptotic and proliferative activity of curcumin on ovarian follicles in mice exposed to whole body ionizing radiation. Toxicol Indus Health. 2012;28:852–63.
31. Rajpert-De Meyts E, Jorgensen N, Graem N, Müller J, CateR L, Skakkebaek NE. Expression of anti-mullerian hormone during normal and pathological gonadal development: association with differentiation of Sertoli and granulosa cells. J Clin Endocrinol Metab. 1999;84:3836-44.
32. Tolikas A, Tsakos E, Gerou S, Prapas Y, Loufopoulos A. Anti-mullerian hormone (AMH) levels in serum and follicular fluid as predictors of ovarian response in stimulated (IVF and ICSI) cycles. Hum Fert. 2011;14:246–53.
33. Visser JA, de Jong FH, Laven JS, Themmen AP. Anti-mullerian hormone: a new marker for ovarian function. Reproduction. 2006;131:1–9.
34. Hansen KR, Knowlton NS, Thyer AC, Charleston JS, Soules MR, Klein NA. A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause. Hum Reprod. 2008;23:699–708.
35. Ibrahim MA, Albahlol IA, Wani FA, Abd-Eltawab Tammam A, Kelleni MT, Sayeed MU, et al. Resveratrol protects against cisplatin-induced ovarian and uterine toxicity in female rats by attenuating oxidative stress, inflammation and apoptosis. Chem Biol Interact. 2021;338:109402.
36. Salvioli S, Sikora E, Cooper EL, Franceschi C. Curcumin in cell death processes: a challenge for CAM of age-related pathologies. Evid Based Comple Alter Med. 2007;4:181-90.
37. Morgan S, Anderson RA, Gourley C, Wallace WH, Spears N. How do chemotherapeutic agents damage the ovary? Hum Reprod Update. 2012;18:525–35.
38. Morgan S, Lopes F, Gourley C, Anderson RA, Spears N. Cisplatin and doxorubicin induce distinct mechanisms of ovarian follicle loss; imatinib provides selective protection only against cisplatin. PLoS One. 2013;8:e70117.
39. Chang EM, Lim E, Yoon S, Jeong K, Bae S, Lee DR et al. Cisplatin induces overactivation of the dormant primordial follicle through PTEN/AKT/FOXO3a pathway which leads to loss of ovarian reserve in mice. PLoS One. 2015;10:0144245.