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Evaluating Effects of Black Carrot Extract on Testicular Carboxylesterase Activity and Oxidative Stress Parameters in Rats Exposed to Bisphenol A

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Abstract

We aimed to evaluate the effects of black carrot extract on testicular carboxylesterase (Ces) activity, malondialdehyde (MDA), glutathione S-transferases (GST) and reduced glutathione (GSH) in male rats exposed to bisphenol A (BPA). Adult Wistar albino male rats were divided into 4 groups as follows (n = 7/group): control, BPA, black carrot and BPA+Black carrot. Testicular Ces, MDA, GST and reduced GSH were analyzed by using a spectrophotometer system. Testicular Ces activity was significantly lower only in the BPA group than the control group ($p < 0.001$). Testicular MDA concentrations were higher only in the BPA group compared with the control group ($p < 0.001$). Reduced GSH level was higher in the black carrot and BPA+Black carrot groups than the control group ($p < 0.05$). GST activity was lower in the BPA

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group in comparison to the control group ($p < 0.05$). In the black carrot and BPA+Black carrot groups, the GST activities were higher than the control group ($p < 0.001$). Our results showed BPA suppresses testicular detoxification activity and increases lipid peroxidation in rats. We observed that black carrot extract has a beneficial effect on the toxic and oxidative stress-related parameters caused by BPA exposure.

***Keywords**:* Bisphenol A; Black carrot; Testis; Carboxylesterase; Oxidative stress; Rat.

Bisfenol A'ya Maruz Kalan Sıçanlarda Siyah Havuç Ekstresinin Testis Karboksilesteraz Aktivitesi ve Oksidatif Stres Parametreleri Üzerindeki Etkilerinin Değerlendirilmesi

Öz

Bu çalışmada Bisfenol A'ya (BPA) maruz kalan erkek sıçanlarda siyah havuç ekstraktının testiküler karboksilesteraz (Ces) aktivitesi, malondialdehit (MDA) düzeyi, indirgenmiş glutatyon (GSH) ve glutatyon S-transferaz (GST) aktiviteleri üzerine etkilerini değerlendirmeyi amaçladık. Yetişkin erkek Wistar albino sıçanlar kontrol, BPA, siyah havuç ve BPA+Siyah havuç olmak üzere 4 gruba ayrıldı ($n = 7$/grup). Testis dokusunda Ces, MDA, indirgenmiş GSH ve GST düzeyleri mikroplaka okuyucu spektrofotometre sistemi kullanılarak analiz edildi. Testis Ces aktivitesi sadece BPA grubunda kontrol grubuna kıyasla önemli düzeyde daha düşüktü ($p < 0.001$). Testis MDA konsantrasyonu sadece BPA grubunda kontrol grubuna kıyasla daha yüksekti ($p < 0.001$). İndirgenmiş GSH düzeyi siyah havuç ve BPA+Siyah havuç gruplarında kontrol grubuna kıyasla daha yüksekti ($p < 0.05$). GST aktivitesi BPA grubunda kontrol grubuna göre daha düşüktü ($p < 0.05$). Siyah havuç ve BPA + Siyah havuç gruplarında GST aktiviteleri kontrol grubuna kıyasla daha yüksekti ($p <0.001$). Sonuçlarımız, BPA'nın sıçan testis dokusunda detoksifikasyon aktivitesini baskıladığını ve lipit peroksidasyonunu arttırdığını gösterdi. Siyah havuç ekstraktının, BPA maruziyetinin neden olduğu toksik ve oksidatif stresle ilişkili parametreler üzerinde yaralı bir etkiye sahip olduğunu gözlemledik.

***Anahtar Kelimeler**:* Bisfenol A; Siyah havuç; Testis; Karboksilesteraz; Oksidatif stres; Sıçan.

1. Introduction

Fruits and vegetables are foods that have invaluable beneficial effects on both nutrition and health with their vitamins, minerals and other natural biological components. These food sources can be consumed as processed products, such as juices, canned foods or jams, and naturally unprocessed products. It has been understood that consuming them as natural as possible, especially unprocessed, is important for their health benefits to emerge as expected. *Carrot or*

Daucus carota is a member of the *Apiaceae* family (also called as *Umbelliferae*). This biennial vegetable can be classified into two groups as the carotene (*Daucus carota ssp. sativus*) and the anthocyanin (*Daucus carota subsp. sativus var. atrorubens*) groups [1]. Although the carotene group is grown in many countries across the world, the anthocyanin group, also known as black carrots, is grown in especially in Turkey, Egypt, Afghanistan and India [2]. Black carrots have a bluish-purple color due to their high anthocyanin levels and may be used as natural food coloring. Moreover, black carrot extracts offer an important alternative to synthetic colorants being a less toxic, more pH- and heat-stable food colorant [3]. Fresh black carrots have high anthocyanin content, which may be about 1750 mg/kg [4]. Additionally, acylated cyanidin-based anthocyanins in the content of black carrot are reported to be mainly cyanidin 3-feruloyl-xylosyl-glucosyl-galactoside (13.5%) and cyanidin 3-synapoyl-xylosyl-glucosyl-galactoside (27.5%) [3, 4]. Anthocyanin derivatives in black carrots have been suggested to have antioxidant activity [5, 6]. In some studies, carrot seed extract has been reported to contribute to reducing reproductive toxicity caused by toxic agents [7, 8].

Bisphenol A (BPA), a xenoestrogen and toxic agent, is the building block in the manufacture of several polycarbonate plastic products such as water tubes, drinking containers, toys and epoxy resins papers [9]. Important sources of BPA exposure in the human population are oral, through inhalation and transdermal [10]. In humans, there is a relationship between BPA levels in the body and health problems. BPA has been defined as a causative agent for various metabolic disorders such as hepatic dysfunction, cancer, type 2 diabetes and infertility [11]. Although scientific data from animal models show the adverse effects of BPA on reproductive function, there is an increasing literature investigating the destructive effects of it on the male reproductive system, but offering heterogeneous and sometimes contradictory findings between the animals and humans [12]. These reports indicate that BPA has complex toxic effects on the reproductive system. One of the negative effects of BPA on the reproductive system, it causes oxidative stress in the testicles and epididymis by stimulating lipid peroxidation and inhibiting antioxidant enzymes [13]. Despite the relatively low oxygen capacity compared to organs such as the brain, which characterize the testicular microenvironment, testes remain vulnerable to the oxidative stress due to high levels of unsaturated fatty acids (especially 20:4 and 22:6) and presence of potentially reactive oxygen species (ROS) [14]. High ROS production in the testes has been reported to cause important changes in the testicular physiological functions that may lead to infertility [15]. The interaction of BPA as a xenoestrogen and toxic agent with carboxylesterase (Ces), a xenobiotic metabolizing enzyme [16], may also play a role in the pathophysiological process associated with reproductive dysfunction. It is suggested that, based on a well-known role of Ces in the detoxification of chemicals and environmental pollutants, it is

possible that Ces in the male genital system may exhibit a similar physiological function capable of modulating dysfunction in the reproductive system against xenobiotic effects [17]. To the best of our knowledge, there has been no report on the effects of black carrot on testicular Ces activity. Therefore, in our study, it was aimed to determine the effects of black carrot on testis Ces activity, as well as malondialdehyde (MDA), glutathione S-transferases (GST) and reduced glutathione (GSH) levels in male rats exposed to BPA.

2. Materials and Methods

2.1. Animal models and experimental protocol

Adult male Wistar albino rats (230 ± 20 g) were obtained from the Experimental Research Center of Fırat University (FUDAM, Elazig, Turkey). The rats were housed under standard light-dark schedule (12h light: 12 h darkness from 19:00), at a standard humidity (55 ± 5%) and constant temperature (21 ± 1 °C). They were fed with ad libitum standard rat diet and fresh tap water. The approval for the experimental protocol was given by the Animal Experiments Local Ethics Committee of Fırat University (Protocol no: 2017/111). National and international laws and policies on experimental animals were cared.

The rats were divided into four groups as Control, BPA, Black carrot and BPA + Black carrot (n=7 for each group). The black carrots, which were thoroughly washed and cleaned, were passed through a juicer. BPA (20 μg/kg) and black carrot juice (4ml/kg) were administered orally to the rats via an orogastric gavage throughout 60 days with 1-day intervals [18, 19]. Black carrot juice was prepared fresh before each application. The rats were sacrificed at the end of 60 days. The testes were dissected from the animals.

2.2. Chemicals

BPA, potassium phosphate buffer (PPB), p-nitrophenyl acetate (PNPA), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithio-bis (2-nitrobenzoic acid (DTNB), bovine serum albumin (BSA) and Bradford reagent were obtained from Sigma (Dorset, UK) unless otherwise stated.

2.3. Tissue homogenization

According to our previous method [20], homogenization of the testicular tissue was performed using a homogenizer (Heidolph RZ 2021, Germany) in a cooled homogenization buffer (pH 7.4 in 0.1 M PPB; 1mM DTT, 0.15M KCl, 1mM EDTA,). After homogenization, the homogenates were transferred into Eppendorf tubes and centrifuged at 16,000 g for 20 minutes at 4 °C (Sigma 2-16K, St. Louis, Missouri). Following this process, the supernatant fraction was removed, and analyzes were performed on these samples.

2.4. Total protein analyses

The total protein level in the supernatant samples was measured using a previously described method [21]. Briefly, 250 μL of Bradford reagent and 5 μL of diluted supernatant (1/4) were added into each microplate wells. After 15-minutes incubation period, absorbance was recorded at 595 nm.

2.5. Determination of testicular total Ces activity

A spectrophotometric method [22] adapted to the microplate reader spectrophotometer system (ThermoTM Varioskan Flash - Thermo Fisher Scientific, Vantaa, Finland) was used to measure the total Ces activity. In the activity analysis, PNPA was prepared in 26 mM ethanol (96%), and this prepared solution was used as substrate. Alterations in absorbance were recorded at 405 nm for 2 minutes at 25 °C.

2.6. MDA, reduced GSH and GST analyses

The testis MDA levels were determined based on relative production of reactive substances of the thiobarbituric acid [23]. The reduced GSH activity was measured by the substance's reaction with DTNB at 412 nm [24].

For determining GST activity, firstly, 20 mM of CDNB was prepared in ethanol (96%), and this chemical was used as substrate solution. 0.002 M reductive GSH solution was used as cofactor in the reaction [25]. 100 µL of the GSH mixture + 10 µL of the supernatant + 100 µL of the PBS (pH 6.5 and 0.1 M) and finally 10 µL of CDNB were transferred into microplate wells. In the following process, change in absorbance was monitored at 344 nm for 2 minutes at 25 °C.

2.7. Statistical analysis

The comparison of the groups was performed using one-way analysis of variance (ANOVA) followed by Tukey test. Statistical significance was accepted as $p < 0.05$.

3. Results

Figure 1 shows the results on the testicular tissue Ces activity. The testicular Ces activity was significantly lower only in the BPA group (812 ± 75 nmol/min/mg protein) than the control group (1430 ± 32 nmol/min/mg protein, $p < 0.001$). There was no significant difference in the Ces activity between the control group and the Black carrot (1661 ± 75 nmol/min/mg protein) and BPA+Black carrot (1573 ± 91 nmol/min/mg protein) groups. Moreover, there was a significant difference in the Ces activity between the BPA group and the BPA+Black carrot group ($p <0.001$).

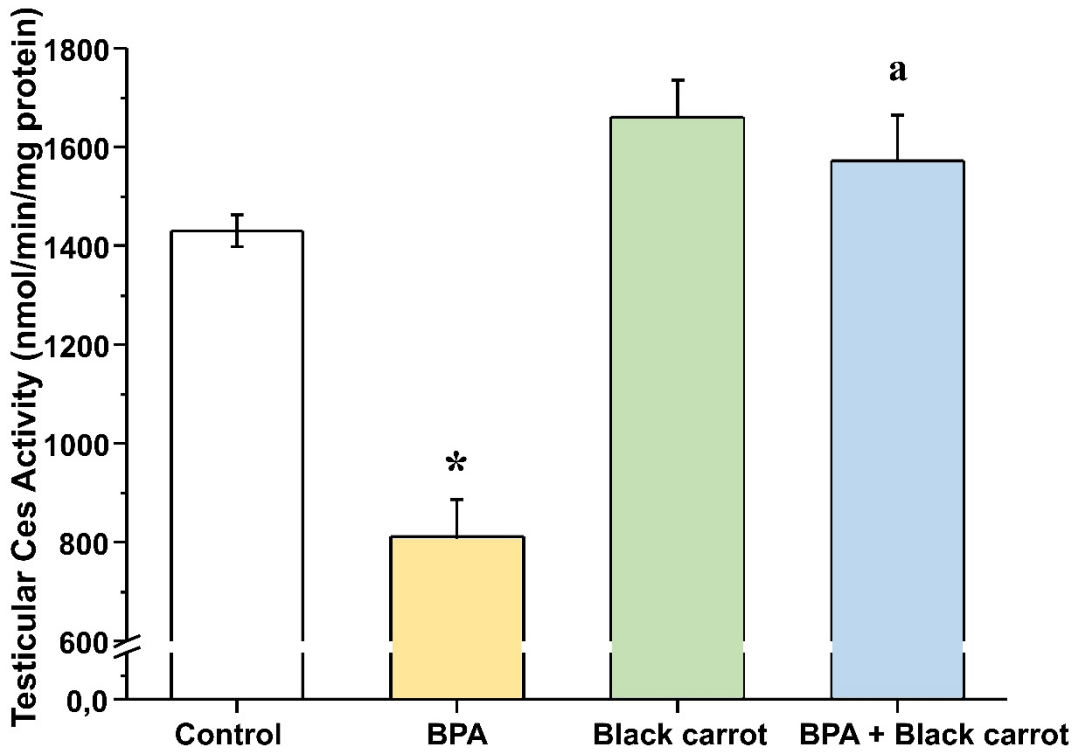

Figure 1: Results of testicular Ces activities for all groups. The data represent mean± SEM. *: $p < 0.001$ compared with the control group, a: $p < 0.001$ compared with the BPA group ($n = 7$ for each group). Ces: Carboxylesterase

Figure 2 shows the results on the testicular tissue MDA level. The MDA concentrations in the testes were higher in the BPA group (605±15 pmol/mg protein) in comparison to the control group (294±12 pmol/mg protein, $p < 0.001$). In the Black carrot (243±38 pmol/mg protein) and BPA+Black carrot (233±45 pmol/mg protein) groups, the MDA concentrations were at the same level as the control group value. Moreover, there was a significant difference in the MDA concentration between the BPA group and the BPA+Black carrot group ($p < 0.001$).

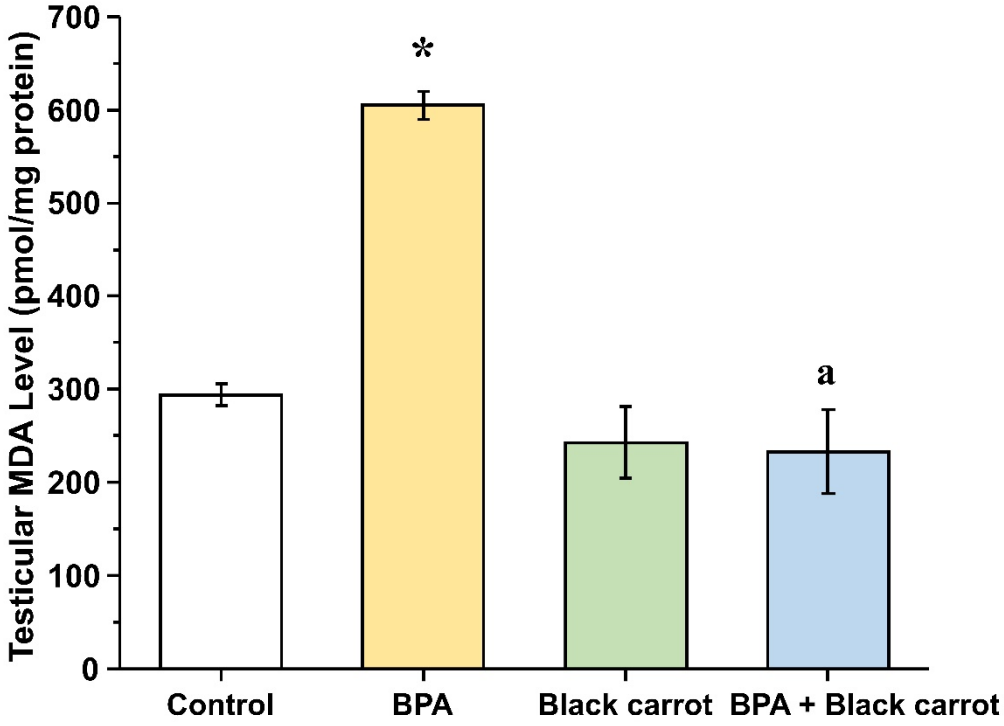

Figure 2: Results of testicular MDA levels for all groups. The data represent mean± SEM. *: $p < 0.001$ compared with the control group, a: $p < 0.001$ compared with the BPA group (n=7 for each group). MDA: Malondialdehyde.

Figure 3 shows the results on the testicular tissue reduced GSH level. The reduced GSH level was higher in the Black carrot (93±4 nmol/mg protein) and BPA+Black carrot (95±5 nmol/mg protein) groups than the control group (67±3 nmol/mg protein, $p<0.05$). In the BPA group (73±4 nmol/mg protein), reduced GSH level did not differ than the control group. However, there was a significant difference in the GSH level between the BPA group and the BPA+Black carrot group ($p < 0.05$).

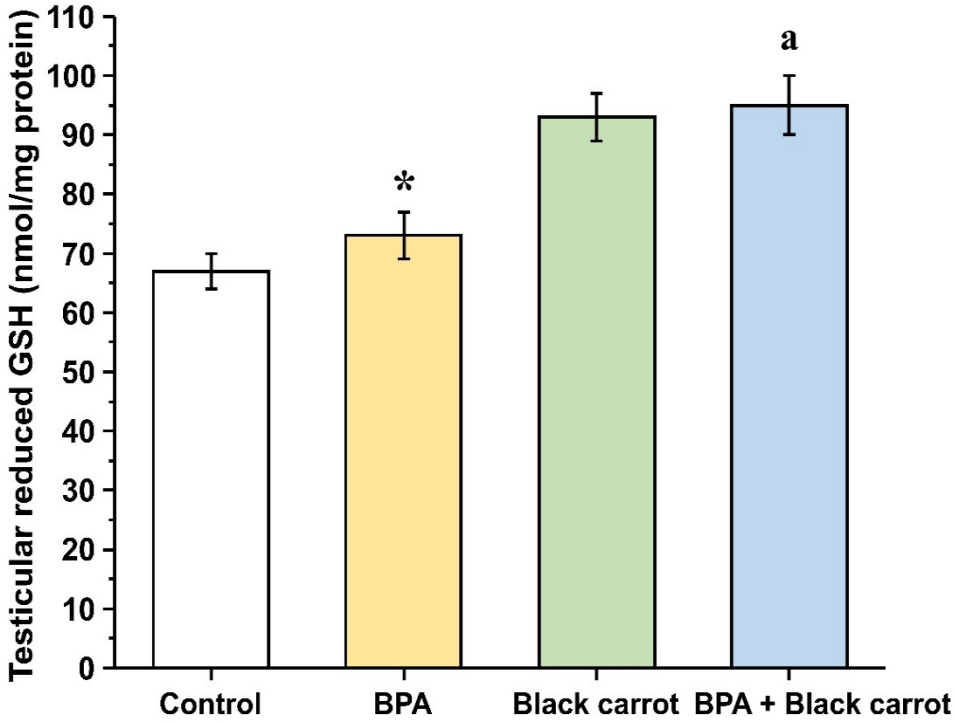

Figure 3: Results of testicular reduced GSH levels for all groups. The data represent mean± SEM. *****: $p < 0.05$ compared with the control group, a: $p < 0.05$ compared with the BPA group (n=7 for each group). GSH: Reduced Glutathione

Figure 4 shows the results on the testicular tissue GST activity. The GST activity was lower in the BPA group (56±3 nmol/min/mg protein) in comparison to the control group (68±3 nmol/min/mg protein, $p < 0.05$). In the Black carrot (88±7 nmol/min/mg protein) and BPA+Black carrot (83±3 nmol/min/mg protein) groups, the GST activities were higher than the control group ($p<0.001$). Moreover, there was a significant difference in the GST activity between the BPA group and the BPA+Black carrot group ($p<0.001$).

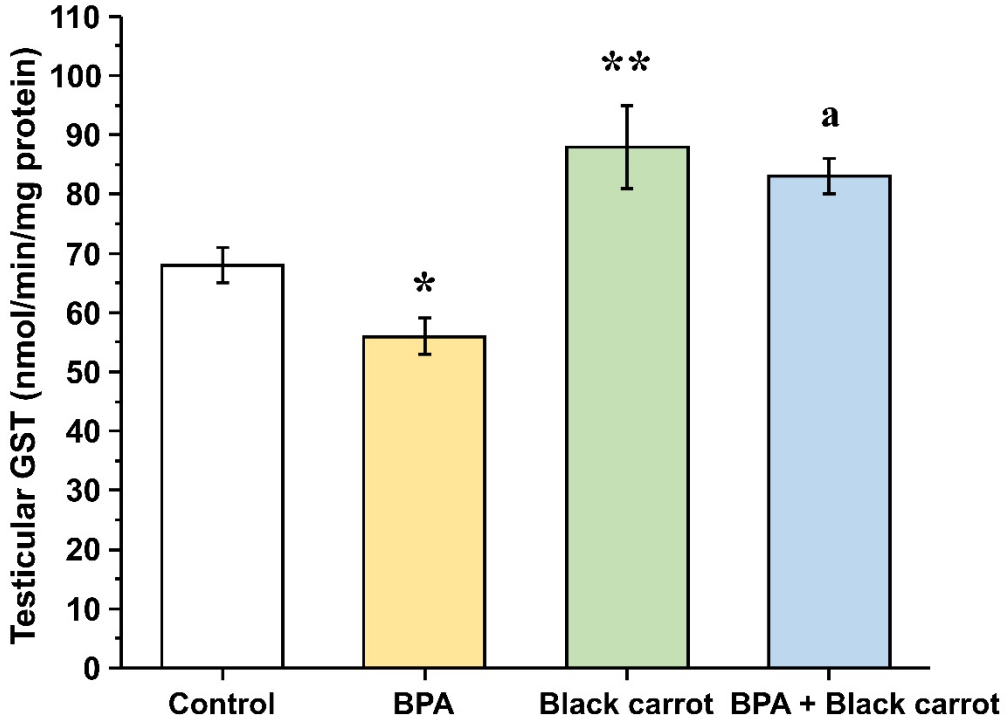

Figure 4: Results of testicular GST activities for all groups. The data represent mean± SEM. *: $p < 0.05$, **: $p < 0.001$ compared with the control group, a: $p < 0.001$ compared with the BPA group ($n = 7$ for each group). GST: Glutathione S-transferases

4. Discussion

This study was based on determination of the effects of black carrot extract on testicular Ces activity, as well as testicular MDA, reduced GSH and GST levels in male rats exposed to BPA. We determined that the testicular Ces activity was significantly lower only in the BPA group in comparison to the control group. The Ces activity of the Black carrot and BPA+Black carrot groups was not significantly different from control group. This result showed that BPA negatively affects Ces activity, a family of the detoxification enzymes, in the testes. These enzymes are a multigene family of serine-dependent enzymes which are localized in the endoplasmic reticulum of cells in the body. Ces enzymes play an substantial role in the metabolism of foreign substrates and endogenous lipids including environmental toxins and drugs [26, 27]. Therefore, a wide range of xenobiotics, drugs and chemicals is determined to alter the activity of Ces enzymes [26]. Comparative assessment of sex-dependent protein expression in the male reproductive tissue of humans and animals has revealed Ces expression in the male reproductive tract. However, very little is known about the functional significance of Ces activity in the male reproductive system. The Ces enzyme family is expressed mainly in the liver and other organs such as the kidney, heart, intestines, lungs, brain and testes [28]. A study reported that, with Ces5a-knockdown, a new Ces gene, male animals exhibited a reduction in fertility [29].

In rats, molinate is an herbicide used on rice, and this chemical covalently reacts with rat Hydrolase A (orthologue of human CES1) in a highly specific manner. This modification causes to remarkably reduced Ces activity in rat liver and testicular tissue in the case that Leydig cells could inhibit mobilization of cholesterol esters required for testosterone synthesis [30]. In the testes, the functional role of Ces is thought to be involved in testosterone biosynthesis and protecting testicular cells from the action of harmful and toxic agents. In rodents, typical "testicular tissue-specific" toxic substances, such as ethane dimethane sulfonate (alkylating antitumor agent) [31], tri-o-cresyl phosphate (plasticizer) [32], inhibit the testicular Ces activity and expression and significantly reduce testosterone levels and testicular functions. BPA has been reported to inhibit xenobiotic metabolizing enzymes and thereby Ces expression in the liver and alter the epigenetic regulation of genes encoding these enzymes [16]. Based on our results regarding Ces, we may state that BPA exposure affects the testicular tissue detoxification process, and black carrot extract modulates the BPA-related toxic effects because the activity of Ces in the BPA + black carrot group was at the same level as the control value.

Growing evidence draws attention to specific flavonoids from phytochemicals present in vegetables and fruits that have beneficial effects on the body [33]. It has been suggested that higher consumption habits of fruit / vegetable-based flavonoids are associated with a lower risk of metabolic and cardiovascular diseases in both gender [34, 35]. Anthocyanins as a group of colorful flavonoids are important biological components that are used as a natural dye sources in the food industry [36]. Pretreatment of rats exposed to cadmium with Hibiscus sabdarrifa L. anthocyanins has been reported to significantly normalize reproductive hormones, especially testosterone levels [37]. Studies on carrot seed extract have been reported to contribute to reducing reproductive problems such as low testosterone caused by toxic agents. In a study on the topic, carrot seed extract was reported to have a beneficial effect profile on decreased spermatogenesis and testosterone level by gentamicin administration [7]. In another study, it was stated that the ethanolic extract of *Daucus carota L.* seeds restores the acetaminophen-induced antiandrogenic effect in male rats and will be useful for drug research for treatment of androgen-related deficiencies due to this important effect [8].

Anthocyanin-rich extracts from fruits and vegetables, as well as purified anthocyanins, reduce lipid peroxidation and increase the body's antioxidant capacity [38]. Cyanidin-based anthocyanin pigments in the black carrots are known to have powerful antioxidant properties [5,6]. In our experiment, we determined that the testicular MDA concentration was higher only in the BPA group. Moreover, the GST activity was lower in the BPA group, but it was higher the black carrot group and the BPA+Black carrot group than the control group. Regarding the GSH levels, we showed that it was higher in the black carrot and the BPA+Black carrot groups in

comparison to the control group. The increased MDA level in the BPA group is an important indicator of oxidative stress. Moreover, GST is an enzyme that has both a detoxifying and antioxidant effect [39]. Therefore, a reduction in the GST activity of the BPA group indicated that this substance reduces the detoxification and antioxidant defense of the testes as both a toxic and oxidant agent. The MDA levels were the same as the control group value in both the black carrot and the BPA+Black carrot groups. There were also significant increases in the GST activity and GSH level of these groups. These results showed that BPA-induced oxidative stress can be reduced by the antioxidant effect of anthocyanins in black carrot extract. It was reported that anthocyanins have a much stronger antioxidant ability than α-tocopherol, vitamin C and some other antioxidants [40, 41]. No negative effects of anthocyanins up to 640 mg/day have been reported in humans [38]. A wide dose range in consumption of fruit or vegetable juices containing anthocyanins may be associated with a low potential for side effects. Additionally, it seems possible that pharmacological compounds to be obtained from such plants may be used in a wide dose range. This feature enriches drug development alternatives from substances in the composition of the black carrot.

5. Conclusions

To the best of our knowledge, this study is the first report to investigate the effects of black carrot extract on changes in testicular Ces activity and lipid peroxidation due to BPA exposure. Based on the results of this study, the Ces and GST activities in the testes decreased, and the MDA level increased as a result of BPA exposure. This indicates that BPA suppresses testicular detoxification activity and increases lipid peroxidation in male rats. We observed that black carrot extract has a curative effect on the toxic and oxidative stress-related parameters caused by BPA exposure. This indicates that black carrot extract may be beneficial in reducing BPA-related negative effects, alone or as a supportive treatment. It may also be stated that the active ingredients in black carrots have a significant potential for use as pharmacological compounds.

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