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Anti-angiogenic and oxidative effects of brilliant blue at different concentrations in chorioallantoic membrane model

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ABSTRACT

Introduction: Artificial food colorings are increasingly used to make the color and appearance of foods more attractive. One commonly used food dye is E133, international-coded brilliant blue (BB). According to the European Food Safety Authority panel results in 2010, it was determined that BB daily remained below the acceptable daily intake (ADI) value for adults and exceeded ADI value for children. The purpose of this study was to investigate the effects of BB on angiogenesis and oxidative stress in the chorioallantoic membrane model.

Materials & methods: In this investigation, fertilized chick eggs free of specific pathogens were used. The eggs that were not fully grown or fertilized were excluded. 50 embryos were distributed into five groups of 10 each. The negative control group was the control group, the positive control group was the bevacizumab group, and three different BB dosages (10^{-4} M, 10^{-5} M, and 10^{-6} M) were identified. At the end of the experiment, anti-angiogenesis scoring, and total antioxidant-oxidant capacity were evaluated.

Results: According to the average score values, the control group had no anti-angiogenic impact, but the bevacizumab group had a strong anti-angiogenic effect (average score 1.1). Furthermore, the 10^{-4} M BB group had a weak anti-angiogenic impact (average score of 0.7), while the 10^{-5} M and 10^{-6} M BB groups had no anti-angiogenic effect (average score of 0.4 and 0.2, respectively). As a result of one-way analysis of variance test, it was seen that BB significantly increased total oxidant capacity and oxidative stress index values in proportion to the increase in dose ($p<0.05$).

Conclusions: BB's oxidant and anti-angiogenic effects indicate that high doses of processed foods containing artificial food dyes carry a risk for viable growth. Since there are not enough studies in the literature showing the oxidant or antiangiogenic effects of BB in chorioallantoic membrane model, the original data we presented in this study are pioneering.

Keywords: brilliant blue, food colors, angiogenesis, oxidative stress, chorioallantoic membrane

INTRODUCTION

Artificial food dyes are increasingly used to make the color and appearance of foods more attractive. Various coloring dyes exist in different colored foods, candies, ice cream-like products, and beverages. One commonly used food dye is E133 international-coded brilliant blue (BB). BB FCF is a chemical compound with the chemical formula $C_{37}H_{34}N_2Na_2O_9S_3$. The bright color makes the food look more attractive. In 1970, the Expert Committee on Food Additives and in 1975, the European Union Scientific Committee on Food (SCF) established an acceptable daily intake (ADI) of 12.5 mg/kg body weight/day for BB. In 1984, SCF re-established ADI at 10 mg/kg body weight/day based on new long-term studies. The dose of ADI has been reduced in available scientific study data due to the risks of genotoxicity, reproductive dysfunction, developmental and long-term toxicity, and carcinogenicity at high doses. According to new studies, ADI value of BB was accepted as 6 mg/kg body weight/day. According to the European Food Safety Authority (EFSA) panel results in 2010, it was determined that BB daily remained below ADI value for adults and exceeded ADI value for children [1].

Disruption of oxidant-antioxidant balance in the direction of oxidative stress greatly affects disease formation in living organisms. If increased oxidative stress cannot be balanced by antioxidant capacity, the resulting oxidative damage leads to structural and functional disorders in cells, tissues, organs, and systems. The antioxidant system includes many enzymes such as catalase, glutathione peroxidase, superoxide dismutase, and other organic compounds. Literature data shows that hundreds of important diseases are triggered due to increased oxidative damage [2, 3].

Chorioallantoic membrane (CAM) is a vascularized extraembryonic membrane consisting of two layers: the chorion and the allantois derived from mesoderm. CAM is highly vascularized, and its blood vessels play a crucial role in

exchanging gases between the embryo and the environment. The chick CAM model is a widely used experimental model in various research fields, including angiogenesis, drug testing, xenografting, and cancer research. It is a low-cost, low-maintenance, and easily accessible in vivo animal model that can be used as an alternative to other mammal experimental models. CAM model is in line with increasing public awareness of animal welfare and ethics in research. CAM model has been particularly useful in studying oncology, tumor metastasis, angiogenesis, and therapeutic response to novel agents [4, 5].

Bevacizumab is a monoclonal immunoglobulin G (IgG) antibody that inhibits the binding of vascular endothelial growth factor-A (VEGF-A) to endothelial cells. Bevacizumab, which reduces tumor development by blocking production of new vasculature, is used to treat a variety of cancers [6].

This widespread use of BB an artificial food coloring raises concerns about health effects, especially for children. Although it is a widely used food dye, lack of sufficient studies in literature showing toxic, oxidant, or antiangiogenic effects of BB increases original value of our research. Purpose of this study was to investigate effects of BB on angiogenesis and oxidative stress in CAM model.

MATERIALS & METHODS

Every phase of the experiment was carried out in accordance with the Animal Welfare Act and the guide for the care and use of laboratory animals. This model does not require animal protocol approval [7]. This research was carried out at the Alanya Aladdin Keykubat University Faculty of Medicine's multidisciplinary laboratory.

Chorioallantoic Membrane

In this investigation, fertilized chick eggs free of specific pathogens were used. The eggs that were not fully grown or fertilized were excluded. 50 embryos were distributed into five groups of ten each. Negative control group was control group, positive control group was bevacizumab group, and three different BB dosages (10^{-4} M, 10^{-5} M, & 10^{-6} M) were identified. On the first day, the fertilized chicken eggs were disinfected with 70% alcohol and placed in the incubator at 37 °C and 60-80% humidity. On the 3rd day of incubation, 4-5 CC of albumen was collected from bottom of each egg. Embryos were returned to the incubator. The window was opened on the fifth day of incubation. The pellets were put on CAM, where vascular branching could be seen via the formed windows. Eggs were put in an upright posture in the incubator. Angiogenesis in CAM was investigated on 6th, 7th, and 8th days of incubation. On the eighth day of incubation, each embryo's albumen was extracted and portioned into eppendorf tubes. Albumen samples collected for testing were stored at -80 °C until utilized.

Anti-Angiogenesis Scoring

The anti-angiogenic impact was assessed through the window. The creation of new vessels from the embryo's primary branches and increased neovascularity have been investigated. The anti-angiogenic impact score was calculated using the scoring system established in earlier studies: 0.5, decreased capillary density but not greater than pellet; 1, small capillary-free space that is not larger than twice the pellet size; 2, capillary-free space around the pellet that is at least twice the pellet size. With data provided by scoring assessment of anti-angiogenesis in embryos, average score values were determined, and anti-angiogenic effects were evaluated using this average score. A score less than 0.5 shows no anti-angiogenic impact, a score between 0.5-1 shows weak anti-angiogenic effect, and scores more than 1 show strong anti-angiogenic effect. Average scores were obtained, as follows:

Average score value=([Number of embryos (score 1)×1]+[Number of embryos (score 2)×2])/Total number of embryos [8].

Measurement of Oxidative Stress Markers

Total oxidant capacity (TOC) and total antioxidant capacity (TAC) were measured with the modified Erel method using a colorimetric commercial kit (Rel Assay, Mega Tip, Gaziantep, Turkey), oxidative stress index (OSI) was calculated by TOC/TAC formula [9, 10].

Statistical Analysis

The average score values based on the scoring technique reported in earlier research [8] were used to assess angiogenesis. The raw data were shown as the mean±standard deviation (SD). The one-way analysis of variance (ANOVA) test was used to compare oxidative stress indicators. Tukey and Duncans post-hoc tests were used to compare the groups. A p-value of less than 0.05 was considered statistically significant.

RESULTS

According to the average score values, the control group had no anti-angiogenic impact, but the bevacizumab group had a strong anti-angiogenic effect (average score 1.1). Furthermore, the 10^{-4} M BB group had a weak anti-angiogenic impact (average score of 0.7), while the 10^{-5} M and 10^{-6} M BB groups had no anti-angiogenic effect (average score of 0.4 and 0.2, respectively) (part A-part F in **Figure 1**).

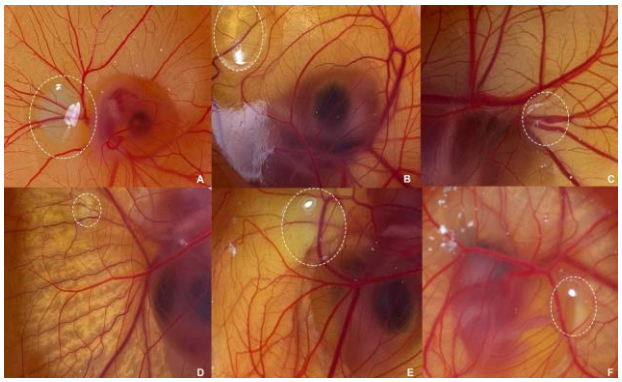

Figure 1. Pellet application on 5th day (A); formation of a vascular bed following free pellet implantation (well-developed vascularity) (B); score 0.5 suppression of vascular bed growth following 10^{-6} M BB pellet implantation (decreased capillary density but not greater than pellet) (C); score 1 suppression of vascular bed growth following 10^{-5} M BB pellet implantation (small capillary-free region and lower capillary density) (D); inhibition of vascular bed growth following pellet implantation with 10^{-4} M BB (capillary-free region around the pellet) (score 2) (E); & capillary-free region around the pellet (score 2 suppression of vascular bed growth following pellet implantation with 10^{-6} M bevacizumab) (F) (Source: Authors' own elaboration)

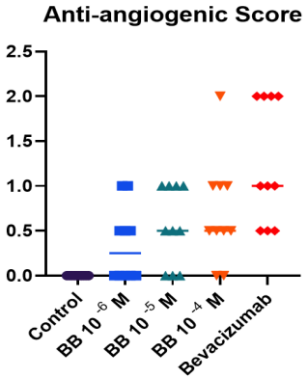

Figure 2. Anti-angiogenic scores of embryos treated with control, bevacizumab, & BB at various dosages are presented using previously established anti-angiogenic scoring method (Source: Authors' own elaboration)

All groups' average scores were calculated and provided (**Figure 2**).

Oxidant-Antioxidant Analyses

As a result of ANOVA test, it was seen that BB significantly increased TOC and OSI values in proportion to increase in dose ($p<0.05$). Biochemical analysis results are given in **Table 1**.

Table 1. Results of oxidant-antioxidant analyses

		n	M	SD	SEM	95% CI for M		p
						LB	UB	
TAC	Pre-proc. control	10	0.97	0.15	0.05	0.87	1.08	<0.01*
	Post-proc. control	10	0.73	0.10	0.03	0.66	0.80	
	Bevacizumab	20	1.45	0.72	0.16	1.12	1.79	
	10^{-4} BB	10	1.10	0.47	0.15	0.76	1.43	
	10^{-5} BB	10	0.76	0.38	0.12	0.48	1.03	
	10^{-6} BB	10	1.54	0.50	0.16	1.18	1.90	
TOC	Pre-proc. control	10	2.72	0.56	0.18	2.32	3.12	<0.01*
	Post-proc. control	10	4.54	0.54	0.17	4.15	4.93	
	Bevacizumab	20	12.61	2.97	0.66	11.22	14.00	
	10^{-4} BB	10	16.92	8.68	2.75	10.71	23.13	
	10^{-5} BB	10	13.65	2.79	0.88	11.65	15.65	
	10^{-6} BB	10	14.76	4.92	1.56	11.24	18.28	
OSI	Pre-proc. control	10	2.85	0.75	0.24	2.31	3.39	<0.01*
	Post-proc. control	10	6.31	1.18	0.37	5.47	7.15	
	Bevacizumab	20	12.50	9.64	2.16	7.99	17.01	
	10^{-4} BB	10	20.14	17.30	5.47	7.77	32.52	
	10^{-5} BB	10	22.79	13.75	4.35	12.95	32.62	
	10^{-6} BB	10	12.06	9.50	3.00	5.26	18.86	

Note. TOC: Total oxidant capacity; TAC: Total antioxidant capacity; OSI: Oxidative stress index; M: Mean; SD: Standard deviation; CI: Confidence interval; LB: Lower bound; UB: Upper bound; BB: Brillant blue, & *Statistically significant difference

Table 2. TAC post-hoc tests

Dependent variable			MD (I-J)	SE	Sig.	95% CI	
						LB	UB
TAC Tukey HSD	Pre-proc. control	Post-proc. control	0.24	0.22	0.89	-0.41	0.89
		B	-0.48	0.19	0.14	-1.04	0.08
		10^{-4} BB	-0.12	0.22	0.99	-0.77	0.53
		10^{-5} BB	0.21	0.22	0.93	-0.44	0.86
		10^{-6} BB	-0.57	0.22	0.12	-1.22	0.08
	Post-proc. control	Pre-proc. control	-0.24	0.22	0.89	-0.89	0.41
		B	-.719*	0.19	0.00	-1.28	-0.16
		10^{-4} BB	-0.36	0.22	0.58	-1.01	0.29
		10^{-5} BB	-0.02	0.22	1.00	-0.68	0.63
		10^{-6} BB	-.806*	0.22	0.01	-1.46	-0.16

Table 2 (continued). TAC post-hoc tests

Dependent variable			MD (I-J)	SE	Sig.	95% CI	
						LB	UB
TAC Tukey HSD	B	Pre-proc. control	0.48	0.19	0.14	-0.08	1.04
		Post-proc. control	.71900*	0.19	0.00	0.16	1.28
		10^{-4} BB	0.36	0.19	0.43	-0.21	0.92
		10^{-5} BB	.694*	0.19	0.01	0.13	1.26
		10^{-6} BB	-0.09	0.19	1.00	-0.65	0.48
	10^{-4} BB	Pre-proc. control	0.12	0.22	0.99	-0.53	0.77
		Post-proc. control	0.36	0.22	0.58	-0.29	1.01
		B	-0.36	0.19	0.43	-0.92	0.21
		10^{-5} BB	0.34	0.22	0.65	-0.31	0.99
		10^{-6} BB	-0.44	0.22	0.35	-1.09	0.21
	10^{-5} BB	Pre-proc. control	-0.21	0.22	0.93	-0.86	0.44
		Post-proc. control	0.02	0.22	1.00	-0.63	0.68
		B	-.694*	0.19	0.01	-1.26	-0.13
		10^{-4} BB	-0.34	0.22	0.65	-0.99	0.31
		10^{-6} BB	-.781*	0.22	0.01	-1.43	-0.13
	10^{-6} BB	Pre-proc. control	0.57	0.22	0.12	-0.08	1.22
		Post-proc. control	.806*	0.22	0.01	0.16	1.46
		B	0.09	0.19	1.00	-0.48	0,65
		10^{-4} BB	0.44	0.22	0.35	-0.21	1.09
		10^{-5} BB	.781*	0.22	0.01	0.13	1.43

Note. TAC TUKEY HSD multiple comparisons; TAC: Total antioxidant capacity; B: Bevacizumab; MD: Mean difference; SE: Standard error; CI: Confidence interval; LB: Lowerbound; & UB: Upper bound

Table 3. TOC post-hoc tests

Dependent variable			MD (I-J)	SE	Sig.	95% CI	
						LB	UB
TOC Tukey HSD	Pre-proc. control	Post-proc. control	-1.82	1.89	0.93	-7.37	3.72
		B	-9.893*	1.63	0.00	-14.69	-5.09
		10^{-4} BB	-14.20*	1.89	0.00	-19.74	-8.66
		10^{-5} BB	-10.93*	1.89	0.00	-16.48	-5.39
		10^{-6} BB	-12.04*	1.89	0.00	-17.58	-6.50
	Post-proc. control	Pre-proc. control	1.82	1.89	0.93	-3.72	7.37
		B	-8.071*	1.63	0.00	-12.87	-3.27
		10^{-4} BB	-12.37*	1.89	0.00	-17.92	-6.83
		10^{-5} BB	-9.110*	1.89	0.00	-14.65	-3.57
		10^{-6} BB	-10.21*	1.89	0.00	-15.76	-4.68
	B	Pre-proc. control	9.893*	1.63	0.00	5.09	14.69
		Post-proc. control	8.071*	1.63	0.00	3.27	12.87
		10^{-4} BB	-4.31	1.63	0.10	-9.11	0.49
		10^{-5} BB	-1.04	1.63	0.99	-5.84	3.76
		10^{-6} BB	-2.15	1.63	0.78	-6.95	2.65
	10^{-4} BB	Pre-proc. control	14.200*	1.89	0.00	8.66	19.74
		Post-proc. control	12.378*	1.89	0.00	6.83	17.92
		B	4.31	1.63	0.10	-0.49	9.11
		10^{-5} BB	3.27	1.89	0.52	-2.28	8.81
		10^{-6} BB	2.16	1.89	0.86	-3.38	7.70
	10^{-5} BB	Pre-proc. control	10.932*	1.89	0.00	5.39	16.48
		Post-proc. control	9.110*	1.89	0.00	3.57	14.65
		B	1.04	1.63	0.99	-3.76	5.84
		10^{-4} BB	-3.27	1.89	0.52	-8.81	2.28
		10^{-6} BB	-1.11	1.89	0.99	-6.65	4.43
	10^{-6} BB	Pre-proc. control	12.041*	1.89	0.00	6.50	17.58
		Post-proc. control	10.219*	1.89	0.00	4.68	15.76
		B	2.15	1.63	0.78	-2.65	6.95
		10^{-4} BB	-2.16	1.89	0.86	-7.70	3.38
		10^{-5} BB	1.11	1.89	0.99	-4.43	6.65

Note. TOC TUKEY HSD multiple comparisons; TAC: Total antioxidant capacity; B: Bevacizumab; MD: Mean difference; SE: Standard error; CI: Confidence interval; LB: Lowerbound; & UB: Upper bound

Post-hoc test results are also shown. **Table 2** shows the TAC post-hoc tests results.

Table 3 depicts the TOC post-hoc tests results.

Table 4. OSI post-hoc tests

Dependent variable			MD (I-J)	SE	Sig.	95% CI	
						LB	UB
OSI Tukey HSD	Pre-proc. control	Post-proc. control	-3.46	4.67	0.98	-17.19	10.27
		B	-9.65	4.05	0.18	-21.54	2.24
		10^{-4} BB	-17.28*	4.67	0.01	-31.02	-3.56
		10^{-5} BB	-19.93*	4.67	0.00	-33.66	-6.20
		10^{-6} BB	-9.21	4.67	0.37	-22.94	4.52
	Post-proc. control	Pre-proc. control	3.46	4.67	0.98	-10.27	17.19
		B	-6.19	4.05	0.65	-18.08	5.70
		10^{-4} BB	-13.83*	4.67	0.05	-27.56	-0.10
		10^{-5} BB	-16.47*	4.67	0.01	-30.21	-2.75
		10^{-6} BB	-5.75	4.67	0.82	-19.48	7.98
	B	Pre-proc. control	9.65	4.05	0.18	-2.24	21.54
		Post-proc. control	6.19	4.05	0.65	-5.70	18.08
		10^{-4} BB	-7.64	4.05	0.42	-19.53	4.25
		10^{-5} BB	-10.28	4.05	0.13	-22.17	1.61
		10^{-6} BB	0.44	4.05	1.00	-11.45	12.33
	10^{-4} BB	Pre-proc. control	17.289*	4.67	0.01	3.56	31.02
		Post-proc. control	13.833*	4.67	0.05	0.10	27.56
		B	7.64	4.05	0.42	-4.25	19.53
		10^{-5} BB	-2.64	4.67	0.99	-16.37	11.09
		10^{-6} BB	8.08	4.67	0.52	-5.65	21.81
	10^{-5} BB	Pre-proc. control	19.933*	4.67	0.00	6.20	33.66
		Post-proc. control	16.477*	4.67	0.01	2.75	30.21
		B	10.28	4.05	0.13	-1.61	22.17
		10^{-4} BB	2.64	4.67	0.99	-11.09	16.37
		10^{-6} BB	10.73	4.67	0.21	-3.00	24.45
	10^{-6} BB	Pre-proc. control	9.21	4.67	0.37	-4.52	22.94
		Post-proc. control	5.75	4.67	0.82	-7.98	19.48
		B	-0.44	4.05	1.00	-12.33	11.45
		10^{-4} BB	-8.08	4.67	0.52	-21.81	5.65
		10^{-5} BB	-1073	4.67	0.21	-24.45	3.00

Note. OSI TUKEY HSD multiple comparisons; TAC: Total antioxidant capacity; B: Bevacizumab; MD: Mean difference; SE: Standard error; CI: Confidence interval; LB: Lowerbound; & UB: Upper bound

Table 4 shows the OSI post-hoc tests results.

DISCUSSION

As a result of the increase in processed food and the increasing prevalence of packaged food products, the exposure and variety of food additives and food dyes are increasing. ADI dose of BB has changed over time according to the possible harmful effects seen as a result of scientific research [1]. The effects of BB, a widely used chemical food dye, on the health of living organisms continue to be a cause for concern.

CAM model is chosen due to its numerous advantages. Among these advantages are faster results than mammalian models [11], direct access to the vascular system without any metabolic or hormonal effects from the mother, macroscopically observing the results of the study, and performing histochemical studies with different sensitivities such as light or electron microscopy. It is appropriate for reverse transcriptase polymerase chain reaction (RT-PCR) studies, where gene expression is determined for samples, the direct effect of applications such as growth factors can be examined without any effect [12], it is easy to access, the cost is low, and it is easy to follow the developmental processes and simplicity of application [4]. Furthermore, because CAM model does not require ethics committee permission and the embryo and membrane structure are kept intact, and it is the closest model to a whole animal experiment. Because of its various benefits, CAM model is employed as one of the most favored approaches as an in vivo angiogenesis model in many investigations [4, 7]. Food additives like acrylamide and monosodium glutamate have been studied for anti-angiogenesis and oxidative stress in CAM model [8, 13]. Accordingly, we conducted our study in CAM model.

VEGF is a crucial chemical regulates neovascularization by initiating angiogenesis [14]. Bevacizumab, a humanized monoclonal antibody derived from Chinese hamsters, binds to and inhibits VEGF-A isoforms [6]. Previous research has revealed that bevacizumab has anti-angiogenic effects in CAM model [8, 15]. We also found that bevacizumab had a considerable anti-angiogenic impact in our investigation. BB caused a greater anti-angiogenic impact at higher dosages in this trial; but this effect was less than that of bevacizumab.

Oxygen-breathing organisms constantly generate oxygen radicals during the functioning of metabolism. Free radicals are atoms or molecules with an unpaired electron in their outermost orbital. The most important free radicals in living organisms are those formed from oxygen. With the reduction of oxygen by gaining an electron each, respectively, superoxide radicals, hydrogen peroxide, and hydroxyl radicals are formed. These reactive oxygen species are radicals and can transform stable structures into radicals. In the face of the constant formation of all these free radicals and the threat to life for the organism, the antioxidant system as a whole works to render them harmless.

The antioxidant system works to prevent the formation of radicals, neutralize the formed radicals, transform the potent radicals into weaker ones, repair the damaged tissue, and increase the antioxidant capacity at the cellular level. Glutathione, glutathione peroxidase, glutathione s-transferase, superoxide dismutase, catalase, paraoxonase, ascorbic acid, beta carotene, tocopherol, melatonin, hemoglobin, ferritin, bilirubin, and transferrin can be counted as important antioxidants in living organisms [16-20]. The antioxidant system and oxidative stress formation contain many components, making it difficult to measure and evaluate biochemically. Thanks to the modified Erel method, a colorimetric total oxidant and antioxidant capacity measurement method, it is possible to evaluate all these components with two general measurements. The OSI calculated with the TOC/TAC ratio excludes reactive oxidant and antioxidant increases. For this reason, OSI values are a very valuable parameter in showing oxidative damage and the risk of oxidative damage-related diseases [21, 22]. Our research results show that BB exposure significantly increases oxidative stress and OSI value in a dose-dependent manner ($p<0.05$). For this reason, one of the mechanisms under the anti-angiogenesis effect of BB, which emerged in our research results, can be considered as an increase in oxidative stress. In pregnancy, it can increase oxidative stress for embryo health; It may be recommended to stay away from chemical food additives, cigarettes, alcohol, ultraviolet, electromagnetic fields, toxic chemical exposures, air pollution, and all similar factors.

Increased oxidative stress can lead to protein fragmentation and aggregation, amino acid modification, DNA mutations, and oxidation of monosaccharides. Oxidative damage can lead to diabetes mellitus, renal failure, cervical cancer, aging, liver damage, Parkinson's, muscular dystrophy, atherosclerosis, and many other chronic fatal diseases. In

addition, antioxidant capacity can be strengthened with suggestions such as eating natural and healthy foods rich in antioxidant vitamins and regular exercise [23-25].

When the data of our study is examined, it is seen that BB may lead to an increase in oxidative stress and angiogenesis inhibition depending on the consumed dose and that mechanisms that can trigger many diseases can be activated in this way. For this reason, considering the widespread use of BB, research on in vivo effects should be continued in a dose-dependent manner.

CONCLUSIONS

BB's oxidant and anti-angiogenic effects indicate that high doses of processed foods containing artificial food dyes carry a risk for viable growth. Since there are not enough studies showing BB's oxidant or antiangiogenic effects in CAM model, the original data presented in this study are pioneering. If similar results are found with new and more comprehensive studies to be done, restricting the consumption of artificial food dyes may be on the agenda. BB's oxidant and anti-angiogenic effects should be revealed more comprehensively with new research. Researching food dyes is very important in public health and preventive medicine. Because food dyes are widely used in the vast majority of processed foods and are consumed by large sections of the public of all ages. BB is one of the most commonly used food dyes. By knowing the oxidant and antiangiogenic effects of BB, it can be ensured that the use of all people, especially newborns, children, pregnant women, and the old humans, who are immune-sensitive, can be restricted and controlled.

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Ethical statement: The authors stated that all necessary ethical rules and legislation in our country and internationally were complied with. Written informed consents were obtained from the participants.

Declaration of interest: No conflict of interest is declared by authors.

Data sharing statement: Data supporting the findings and conclusions are available upon request from the corresponding author.

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