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The effect of tartrazine on angiogenesis and oxidative stress in the chorioallantoic membrane model

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Abstract

Tartrazine is commonly preferred as a coloring agent in non-alcoholic beverages, fruit juices, jellies, cereals, and soups. This study aims to investigate the effects of tartrazine exposure on anti-angiogenesis and the oxidant-antioxidant balance.

Three different tartrazine dose, a bevacizumab, and an empty pellet used to evaluate anti-angiogenic effects on the chorioallantoic membrane (CAM) model. Fluid samples were collected for measurements of total antioxidant capacity (TAC) and total oxidant status (TOS), from which the oxidative stress index (OSI) was calculated.

The control group and 10^{-6} M tartrazine group had no anti-angiogenic impact, but the bevacizumab group had a strong anti-angiogenic effect. Furthermore, the 10^{-4} M and 10^{-5} M tartrazine groups had a weak anti-angiogenic effect. The levels of TOS increase with tartrazine consumption. TAC values were highest in the 10^{-6} M tartrazine group and lowest in the 10^{-5} M tartrazine group. Moreover, OSI values have increased in the 10^{-4} M tartrazine group, 10^{-5} M tartrazine group, and 10^{-6} M tartrazine group compared to control group.

This study demonstrates that tartrazine exposure leads to dose-dependent increases in oxidative stress and, in parallel, exhibits dose-dependent anti-angiogenic effects. For this reason, it is recommended to be careful when consuming products containing tartrazine.

Introduction

Food additives are substances not typical consumed as food and are not food ingredients. They are frequently preferred in processed foods to increase nutritional value and durability and change color and flavor (Lindsay, 2007). According to the findings of many scientific studies, food additives have raised adverse effects on metabolism (Cox et al., 2021). These negative effects have caused the public's interest in the content of the food (Carocho et al., 2015).

Packaged food products is often associated with the product's color, taste and safety (Sigurdson et al., 2017). Food colorants are pigments and dyes used in foods and food derivatives. The primary purpose of food colorants, which are generally used to enhance the food's visual appeal, is to attract the customer's attention and increase the aesthetic appeal. Many natural or synthetic substances can be used as food colorants (Kaya et al., 2021).

Azo colorants are frequently used in the food industry, especially for their color stability. Tartrazine is one of the most commonly used colorants (Ardern & Group, 1996). Tartrazine is a water-soluble synthetic azo dye that colors the food yellow (Mahmoud et al., 2020). Tartrazine is used in many ready-to-eat foods, such as carbonated drinks, fruit juices, sodas, and cakes. Tartrazine's EC number is E 102. Tartrazine is mainly 3-carboxy-5-hydroxy-1-(4'-sulfonatophenyl)-4-(4'-sulfonatophenylazo)-H-pyrazole-3-carboxylate.
Tartrazine has a molecular formula of $C_{16}H_9N_4Na_3O_9S_2$. (Rovina et al., 2017). Scientific studies support that tartrazine may have toxic effects with increased daily intake. Studies on guinea pigs show that toxic effects may occur even at low doses (da Silva & Fracacio, 2021). Tartrazine has also been found to cause hypersensitivity reactions (Elhkim et al., 2007). Accordingly, concerns arise about the widespread use of tartrazine.
Alternative in vivo models, such as chorioallantoic membrane (CAM), is preferred due to their many advantages. It attracts attention due to its advantages in time, low cost, and ease of application (Garcia et al., 2021). During embryonic development, the chorion layer and allantois fuse to form the chorioallantoic membrane (CAM). Vascular networks are rich in capillary blood vessels and are developed to ensure gas exchange. The importance of angiogenesis mechanisms in cancer development is known. Since it is possible to detect the results of the tumor growth research quickly visually, it enables the examination of tumor growth and metastasis angiogenic molecules. It is chosen because it is relatively simple, allowing the screening of the pharmacological sample range quickly. The CAM model does not require administrative procedures, especially for ethics committee approval (Ribatti, 2016). Since tartrazine is a widely used artificial food dye, its potential risks to living things' health are a significant source of concern. This study aims to investigate the effects of tartrazine exposure on anti-angiogenesis and the oxidant-antioxidant balance.

Materials and Methods

Since the CAM model was used, all experimental stages were carried out in accordance with the Animal Welfare Act. There was no need for an experimental animal ethics committee for CAM studies.

Chicken chorioallantoic membrane model

50 fertilized chicken eggs were divided into 5 groups, with 10 fertilized eggs in each group (Table 1). On the first day of incubation, all fertilized chicken eggs were disinfected with 70% alcohol and then placed in the incubator at 37°C and humidity between 60-80%. The eggs were set to rotate every two hours. On the third day of incubation, 5 cc of fluid was extracted from each egg. Following this procedure, the eggs were placed in the incubator. On the fifth day of incubation, an empty pellet and tartrazine were prepared in three doses as 10^{-4} M, 10^{-5} M, and 10^{-6} M and applied through the openings created in the eggshells. The windows were covered with stretch film to protect the eggs from the external environment. Eggs were placed in the incubator vertically. Angiogenesis was examined on the 6th, 7th, and 8th days of incubation, and on the 8th day, fluid was taken from each egg and portioned into Eppendorf tubes. The fluid samples were stored at -80 °C.

Table 1. Design of the study groups.

Group	Number of Embryos (n)	Drug Concentration
Control	10	Stock solution only
Tartrazine	10	10^{-4} M
Tartrazine	10	10^{-5} M
Tartrazine	10	10^{-6} M
Bevacizumab	10	10^{-6} M
Total	50	

***** Drug concentration is the concentration of tartrazine or Bevacizumab in the stock solution. (M: Molar)

Anti-angiogenesis scoring

The score indicating the anti-angiogenic effect was applied according to the scoring principle described in previous studies (Sozen et al., 2022).

In evaluating anti-angiogenesis in embryos, mean score values were derived from the data obtained by scoring the anti-angiogenic effects. According to this scoring system, scores less than 0.5 indicate no anti-angiogenic effect, scores between 0.5 and 1 indicate a weak anti-angiogenic effect, and scores greater than 1 indicate a strong anti-angiogenic effect. The number of eggs receiving a score of 1 or 2 was used to calculate the mean score using the following formula (Table 2).

Mean score value = [(Number of Eggs (score 1) x 1) + (Number of Eggs (score 2) x 2)] / Total Number of Eggs (Sozen et al., 2023).

Table 2. Anti-angiogenic score values

Score	Effect	Observation/ Explanation
0	None	Normal embryo development
0.5	Weak	No capillary-free area, reduced capillary density smaller than pellet
1	Moderate	Decreased capillary density in the specific area or small capillary-free area, and this area is less than 2 times the pellet.
2	Strong	The avascular area around the pellet is more than 2 times the pellet

***** Values obtained by visual evaluation and anti-angionesis scoring.

Total antioxidant capacity

At the end of the 8th day, the liquids transferred to tubes and stored at -80 C. Total antioxidant capacity (TAC) analysis was performed with commercially available kits from Relassay, Turkey. TAC measurement was performed by an automated measurement method, resulting a color change in the ABTS radical (3-ethylbenzothiazollin-6-sulfonic acid) based on the addition of the liquid sample to the medium and the

antioxidant effect of the added sample. The results were expressed in mmol Trolox equivalent/L (Etli et al., 2021).

Total oxidant capacity

At the end of day 8, TOC analysis was performed on the samples. The fluids were transferred to tubes and stored at -80°C until analysis, was performed with commercially available kits from Relassay. The TOC values were measured based on the oxidation reaction. Oxidants oxidized the ferrous ion-o-dianisidine complex to ferric ion. The ferric ion formed a colored complex with xylenol-orange in an acidic medium. The color intensity, measurable spectrophotometrically is related to the sample's total amount of oxidant molecules. The measurement method was calibrated with hydrogen peroxide, and the results were expressed in micromolar hydrogen peroxide equivalents per liter (µmol H_2O_2 equivalents/L) (Savas et al., 2017).

Oxidative stress index

The ratio of TOC to TAC was considered oxidative stress index (OSI). The OSI was calculated as the ratio of TAC measurements to TOC measurements. The OSI value allows a two-way evaluation by excluding reactive increases in the oxidant-antioxidant balance. The results were expressed in arbitrary units (AU). OSI = TOC/TAC (Gunizi et al., 2022; Savran et al., 2019).

Statistical analysis

SPSS 26 (USA) package program was used for statistical analyses. Raw values obtained from all analyses were presented as the mean ± standard error of the groups. In group comparisons, one-way analysis of variance (ANOVA) and multiple comparison analysis tests were used for pairwise comparisons between groups to express the difference, if any, between the groups in the levels of the investigated variable of tartrazine. TUKEY HSD test was used for posthoc comparision. The significance level was accepted as 0.05 in all tests of the study.

Results

According to the average score values, the control group and 10^{-6} M tartrazine group had no anti-angiogenic impact (average score of 0.3), but the bevacizumab group had a strong anti-angiogenic effect (average score of 1.2). Furthermore, the 10^{-4} M and 10^{-5} M tartrazine groups had a weak anti-angiogenic effect (average score of 0.9 and 0.6, respectively). (Figure 1 A-F). All groups' calculated and average scores were provided (Figure 2).

Biochemical measurements were made in triplicate. The TAC level of the 10^{-6} M tartrazine group at different concentrations increased statistically

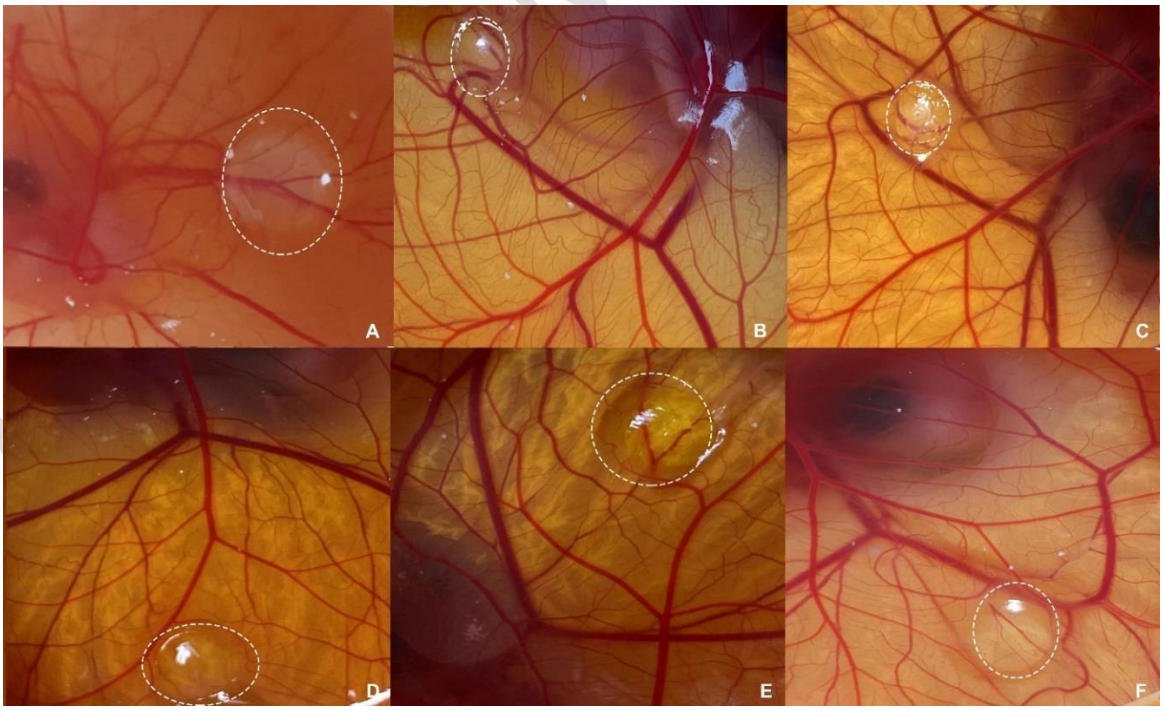

Figure 1. Anti-angiogenic effects of pellets. A. Pellet application on the fifth day of the experiment. B. The formation of a vascular bed following free pellet implantation (well-developed vascularity). C. Score 0.5 suppression of vascular bed growth following 10^{-6} M tartrazine pellet implantation (decreased capillary density but not greater than pellet). D. Score 1 suppression of vascular bed growth following 10^{-5} M tartrazine pellet implantation (small capillary-free region and lower capillary density). E. Inhibition of vascular bed growth following pellet implantation with 10^{-4} M tartrazine (Capillary-free region around the pellet) (Score 2). F. Capillary-free region around the pellet (Score 2 suppression of vascular bed growth following pellet implantation with 10^{-6} M Bevacizumab).

significant, while the 10^{-4} M and 10^{-5} M tartrazine groups increased at a lower rate than the 10^{-6} M tartrazine group ($p<0.05$). The highest TAS value was observed in the 10^{-6} M tartrazine group, while the lowest TAC value was observed in the 10^{-5} M tartrazine group (Table 3 and 4).

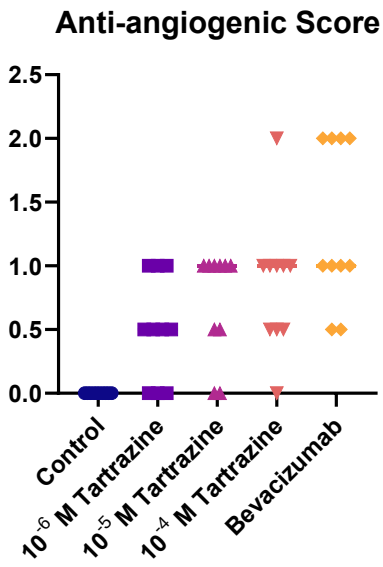

Figure 2. Anti-angiogenic scores of embryos. Anti-angiogenic scores of embryos treated with control, Bevacizumab, and Tartrazine at various dosages are presented using the previously established anti-angiogenic scoring method.

Although TOC values increased in 10^{-4} M, 10^{-5} M, and 10^{-6} M Tartrazine groups compared to control group ($p<0.05$), the highest increase was observed in the 10^{-4} M tartrazine group. According to the results obtained, TOC level increased between the experimental groups with tartrazine consumption at different concentrations, 10^{-4} M had the highest TOC value, while the lowest TOC value was observed in the 10^{-6} M tartrazine group (Table 3 and 5).

According to the results, OSI increased between the experimental groups with tartrazine consumption at different concentrations.10^{-4} M tartrazine group had the highest OSI value. According to OSI values, there is a significant difference between control, 10^{-4} M, 10^{-5} M, and 10^{-6} M groups. Compared to control group, OSI values increased in 10^{-4} M, 10^{-5} M, and 10^{-6} M tartrazine groups. It was observed that OSI was highest in the 10^{-4} M tartrazine group (19.17±12.10), the mean of the 10^{-4} M tartrazine group was higher than the mean of the 10^{-5} M Tartrazine group and the mean of the 10^{-4} M tartrazine group was higher than the mean of the 10^{-6} M tartrazine group (10.68±9.27). The highest increase is observed in the 10^{-4} M tartrazine group (Table 3 and 6).

Discussion

Food additives improve color, flavor, strength, and texture. Food additives are divided into different groups, such as preservatives, nutritional additives, coloring agents, flavor enhancers, and textural enhancers (Carocho et al., 2015). Tartrazine is a yellow-colored, water-soluble powder azo dye (Rovina et al., 2017). Nowadays, exposure to food additives is increasing due to the increase in ready-to-eat food consumption, and it requires more attention to food additives.

The CAM model is one of the most advantageous experimental models for studying embryonic development. Embryo development of the chicken takes 21 days. Chicken embryo development occurs in a shorter period of time compared to developed mammals. For this reason, chicken embryo models allow faster results in embryo experiments. The CAM model is very advantageous for angiogenesis and antiangiogenesis studies because the vessels can be

Table 3. Results of total oxidant-antioxidant analyses

						95% Confidence Interval for Mean				Anova
		n	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum	p
TAC Unit: mmol/L	Control	10	1.03	0.22	0.07	0.88	1.19	0.71	1.49	<0.01
	Bevacizumab	10	1.18	0.29	0.09	0.98	1.39	0.48	1.46	
	10^{-4} M Tartrazine	10	1.14	0.45	0.14	0.82	1.47	0.42	1.91	
	10^{-5} M Tartrazine	10	0.80	0.24	0.08	0.63	0.97	0.51	1.26	
	10^{-6} M Tartrazine	10	1.66	0.57	0.18	1.26	2.07	0.59	2.36	
TOC Unit: µmol/L	Control	10	3.69	1.21	0.38	2.82	4.55	2.14	6.11	<0.01
	Bevacizumab	10	13.12	2.81	0.89	11.11	15.13	9.17	18.52	
	10^{-4} M Tartrazine	10	18.64	7.79	2.46	13.06	24.21	11.30	32.89	
	10^{-5} M Tartrazine	10	14.35	3.69	1.17	11.71	16.99	9.42	22.11	
	10^{-6} M Tartrazine	10	13.89	3.26	1.03	11.56	16.22	10.57	21.10	
OSI =TOC/ TAS	Control	10	3.78	1.71	0.54	2.56	5.00	1.91	7.37	<0.01
	Bevacizumab	10	12.14	5.35	1.69	8.31	15.97	7.57	25.20	
	10^{-4} M Tartrazine	10	19.18	12.10	3.83	10.52	27.83	5.93	49.48	
	10^{-5} M Tartrazine	10	19.01	6.14	1.94	14.62	23.40	12.81	26.96	
	10^{-6} M Tartrazine	10	10.68	9.28	2.93	4.04	17.31	5.79	35.76	

* The mean, standard deviation, and standard error values for total antioxidant capacity (TAC), total oxidant capacity (TOC), and oxidative stress index (OSI) for each group are shown in Table 3.

Table 4. Results of TAC post hoc tests

TAC TUKEY HSD Multiple Comparisons							
Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
TAC	Control	Bevacizumab	-0.15	0.17	0.90	-0.63	0.33
		10^{-4} M Tartrazine	-0.11	0.17	0.97	-0.59	0.37
		10^{-5} M Tartrazine	0.23	0.17	0.64	-0.25	0.71
		10^{-6} M Tartrazine	-.63*	0.17	0.00	-1.11	-0.15
	Bevacizumab	Control	0.15	0.17	0.90	-0.33	0.63
		10^{-4} M Tartrazine	0.04	0.17	1.00	-0.44	0.52
		10^{-5} M Tartrazine	0.38	0.17	0.17	-0.10	0.86
		10^{-6} M Tartrazine	-.48*	0.17	0.05	-0.96	0.00
	10^{-4} M Tartrazine	Control	0.11	0.17	0.97	-0.37	0.59
		Bevacizumab	-0.04	0.17	1.00	-0.52	0.44
		10^{-5} M Tartrazine	0.34	0.17	0.27	-0.14	0.82
		10^{-6} M Tartrazine	-.52*	0.17	0.03	-1.00	-0.04
	10^{-5} M Tartrazine	Control	-0.23	0.17	0.64	-0.71	0.25
		Bevacizumab	-0.38	0.17	0.17	-0.86	0.10
		10^{-4} M Tartrazine	-0.34	0.17	0.27	-0.82	0.14
		10^{-6} M Tartrazine	-.86*	0.17	0.00	-1.34	-0.39
	10^{-6} M Tartrazine	Control	.63*	0.17	0.00	0.15	1.11
		Bevacizumab	.48*	0.17	0.05	0.00	0.96
		10^{-4} M Tartrazine	.52*	0.17	0.03	0.04	1.00
		10^{-5} M Tartrazine	.86*	0.17	0.00	0.39	1.34

*The mean, standard deviation, and standard error values for total antioxidant capacity (TAC), for each group are shown in Table 4.

seen directly, and the molecule's effects are direct to the vessels. The vessels are not affected by the conditions or reactions of the body (Ribatti, 2014). For these reasons this study is carried out in the CAM model.

El-Sakhawy et al. showed that exposure to tartrazine at varying doses causes toxicity in different organs. Tartrazine causes edema, blood vessel occlusion, cell damage in the cerebellum, and blood vessel problems in the salivary glands; hemorrhage and desquamation in the kidneys were observed in mice. These changes were exacerbated as the dose of tartrazine increased. It was also reported that tartrazine caused fetal body weight and length decrease, skeletal malformations, and hepatic and renal damage in the embryo. As a result, tartrazine caused teratogenic effects (Hashem et al., 2019). According to another scientific study, it was observed that tartrazine exposure to chicken embryos at the organogenesis stage in the chick embryo increased the level of DNA damage and increased the apoptosis in the liver and kidneys (El-Borm et al., 2020). A study examining tartrazine's effects on neural tube defects showed that tartrazine caused neural tube defects in the chicken embryo development model (Ovalioglu et al., 2020). Some food additives have anti-angiogenic effects. In accordance with these literature in the present study it is found that tartrazine caused an anti-angiogenic effect dose-dependently.

Providing oxidant and antioxidant balance is very important for living organisms. Many factors determine this balance. Determining TAC, TOC, and OSI is essential

Table 5. Results of TOC post hoc tests

TOC TUKEY HSD Multiple Comparisons							
Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
TOC	Control	Bevacizumab	-9.43*	1.94	0.00	-14.95	-3.92
		10^{-4} M Tartrazine	-14.94*	1.94	0.00	-20.47	-9.43
		10^{-5} M Tartrazine	-10.66*	1.94	0.00	-16.18	-5.14
		10^{-6} M Tartrazine	-10.20*	1.94	0.00	-15.72	-4.69
	Bevacizumab	Control	9.43*	1.94	0.00	3.92	14.95
		10^{-4} M Tartrazine	-5.51	1.94	0.05	-11.03	0.01
		10^{-5} M Tartrazine	-1.23	1.94	0.97	-6.74	4.29
		10^{-6} M Tartrazine	-0.77	1.94	0.99	-6.29	4.75
	10^{-4} M Tartrazine	Control	14.94*	1.94	0.00	9.43	20.47
		Bevacizumab	5.51	1.94	0.05	-0.01	11.03
		10^{-5} M Tartrazine	4.29	1.94	0.20	-1.23	9.80
		10^{-6} M Tartrazine	4.74	1.94	0.12	-0.77	10.26
	10^{-5} M Tartrazine	Control	10.66*	1.94	0.00	5.14	16.18
		Bevacizumab	1.23	1.94	0.97	-4.29	6.74
		10^{-4} M Tartrazine	-4.29	1.94	0.20	-9.80	1.23
		10^{-6} M Tartrazine	0.46	1.94	1.00	-5.06	5.97
	10^{-6} M Tartrazine	Control	10.20*	1.94	0.00	4.69	15.72
		Bevacizumab	0.77	1.94	0.99	-4.75	6.29
		10^{-4} M Tartrazine	-4.74	1.94	0.12	-10.26	0.77
		10^{-5} M Tartrazine	-0.46	1.94	1.00	-5.97	5.06

*The mean, standard deviation, and standard error values for total oxidant capacity (TOC) for each group are shown in Table 5.

Table 6. Results of OSI post hoc tests

OSI TUKEY HSD Multiple Comparisons							
Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
OSI	Control	Bevacizumab	-8.36	3.47	0.13	-18.23	1.51
		10^{-4} M Tartrazine	-15.39*	3.47	0.00	-25.26	-5.52
		10^{-5} M Tartrazine	-15.23*	3.47	0.00	-25.10	-5.36
		10^{-6} M Tartrazine	-6.90	3.47	0.29	-16.77	2.97
	Bevacizumab	Control	8.36	3.47	0.13	-1.51	18.23
		10^{-4} M Tartrazine	-7.03	3.47	0.27	-16.90	2.84
		10^{-5} M Tartrazine	-6.87	3.47	0.29	-16.74	3.00
		10^{-6} M Tartrazine	1.46	3.47	0.99	-8.41	11.33
	10^{-4} M Tartrazine	Control	15.39*	3.47	0.00	5.52	25.26
		Bevacizumab	7.03	3.47	0.27	-2.84	16.90
		10^{-5} M Tartrazine	0.16	3.47	1.00	-9.71	10.03
		10^{-6} M Tartrazine	8.50	3.47	0.12	-1.37	18.37
	10^{-5} M Tartrazine	Control	15.23*	3.47	0.00	5.36	25.10
		Bevacizumab	6.87	3.47	0.29	-3.00	16.74
		10^{-4} M Tartrazine	-0.16	3.47	1.00	-10.03	9.71
		10^{-6} M Tartrazine	8.33	3.47	0.13	-1.54	18.20
	10^{-6} M Tartrazine	Control	6.90	3.47	0.29	-2.97	16.77
		Bevacizumab	-1.46	3.47	0.99	-11.33	8.41
		10^{-4} M Tartrazine	-8.50	3.47	0.12	-18.37	1.37
		10^{-5} M Tartrazine	-8.33	3.47	0.13	-18.20	1.54

*The mean, standard deviation, and standard error values for oxidative stress index (OSI) for each group are shown in Table 6.

because they can show the relevant components together. TOC, TAS and OSI measurements are very valuable and standard parameters to evaluate the oxidant and antioxidant system in general (Ovalioglu et al., 2020). In our study, comprehensive and valuable data were obtained as the oxidant and antioxidant system was evaluated with TOC, TAS and OSI measurements. The oxidative stress causes the cell and tissue damage. Tartrazine caused a decrease in antioxidant parameters and a dose-dependent increase in oxidant parameters. The oxidant values were highest in the 10^{-4} M tartrazine group, and the oxidant values were lowest in the 10^{-6} M tartrazine group, among the tartrazine groups. It was determined that tartrazine caused an increase in oxidative stress, and the average oxidant value increased in direct proportion to the dose. The decreases in antioxidant values and increases in oxidant values were observed due to increasing substance concentration. As a result, tartrazine exposure during pregnancy is a threat because it increases oxidative damage during embryo development and organ formation. It is recommended to increase the quality of life by avoiding a ready-made food diet and preferring fresh and natural products to avoid exposure to artificial food dyes, including tartrazine.

Conclusion

The oxidant and anti-angiogenic effects of tartrazine indicate that high doses of processed foods containing artificial food dyes carry a risk for viable embryo growth. Since there are not enough studies showing tartrazine's oxidant or antiangiogenic effects on CAM model, the original results we presented in this study are contributes novel data. The oxidant and anti-angiogenic effects of tartrazine should be comprehensively revealed with new research.

Author Contributions

Conceptualization: MES and HBS, Data Curation: HBS, Formal Analysis: MES and HBS, Investigation: MES, HBS, and ED, Methodology: MES and HBS, Project Administration: MES, Resources: HBS, Supervision: MES and HBS, Visualization: MES and HBS, Writing -original draft: MES, HBS and ED,

Writing -review and editing: MES, HBS and ED.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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