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Effect of castor oil on esophageal stricture in rats and expression of ST-2, neopterin proteins in corrosive burn model

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Corrosive esophageal burn (CEB) is a disease with high mortality and morbidity rates. The aim of this study was to determine the efficacy of castor oil, in preventing stricture development at the experimental CEB model. In addition to studying standard histopathological damage data, neopterin, IL-33, and ST-2 proteins were also studied for the first time. Fifty Wistar-Albino rats were divided into randomized 5 groups. [Sham group (G1) (n:10), Control group (G2) (n:10), Early Stage Topical Application group (G3)(n:10), Late Stage Topical Application group (G4) (n:10), Oral Application group (G5) (n:10)]. Weight measurement, esophageal length, histopathological damage score (HDS) and total stenosis score (TSS), tissue caspase-3 and VEGF staining, tissue hydroxyproline (HYP), blood TNF-alpha, IL-6, IL-33, Neopterin, and ST-2 levels were measured. In the castor oil application groups, weight gain was observed, the acute phase reaction decreased, submucosal/tunica muscularis fibrosis (TMF) and muscularis mucosal damage (MMD) were reduced, and TSS and HDS decreased. While no significant difference was detected in the ST-2 protein, which was used for the first time in this study model, a significant increase in neopterin protein was observed in the application groups. Results indicate the nutritional contribution of castor oil, as well as its tissue healing and esophageal stricture-preventing efficacy at histopathological and immune-histochemical levels.

Keywords Corrosive esophageal burn, Esophageal stricture, Castor Oil, ST-2, IL-6, Neopterin

Abbreviations

CEB	Corrosive esophageal burn
HDS	Histopathological damage score
HYP	Tissue hydroxyproline
ST-2	Suppression of tumorigenicity-2 protein
SF	Submucosal fibrosis
MMD	Muscularis mucosa damage
TMF	Tunica muscularis fibrosis
TSS	Total stenosis score

Synopsis

In this study, the effectiveness of castor oil in the treatment of corrosive esophageal burns was evaluated for the first time in the literature. Additionally, for the first time in this model, Neopterin, ST-2, and IL-6 proteins were studied in relation to inflammation, stricture, and tissue healing.

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Corrosive esophageal burn (CEB) is a disease with high mortality and morbidity rates, especially in children under the age of 5 years. According to World Health Organization data, 5–15 thousand pediatric corrosive burns require emergency admission in America[1]. In studies conducted in Turkey, it has been found that 3.3–28.1% of child poisonings are due to corrosive substance ingestion[2]. The average mortality rate following oral corrosive ingestion has been determined to be 4.1% in previous studies[3].

After oral corrosive ingestion, tissue damage occurs in three phases. The acute necrotic phase, occurring in the first 1–3 days, involves hemorrhage and thrombosis, followed by local necrosis and angiogenesis. The subacute phase initiates the proliferative stage, characterized by ulceration, mucosal sloughing, fibroblast colonization, and granulation, and lasts 10–14 days. The third stage is the scarring phase, which typically commences approximately three weeks after ingestion and lasts approximately to 3–6 months[3]. The primary goal of treatment is to prevent complications, which can have a high early mortality rate, such as aspiration pneumonia, upper gastrointestinal bleeding, esophageal perforation, mediastinitis, and the development of strictures and carcinoma in the later stages[4].

The most common complication was stricture formation on CEB. Endoscopic dilation was performed as the initial treatment. Preventing stricture is the ideal goal in the treatment of CEB; however, there is still no accepted treatment protocol in current practice[3].

Castor oil is a plant extract derived from Ricinus communis that has been widely used in traditional medicine since ancient times. In the contemporary medical literature, castor oil has been observed to exhibit antifungal, antiviral, and antimicrobial activities[5–7], as well as antioxidant, hepatoprotective, antidiabetic, wound healing, and bone regeneration effects[8]. In current treatment approaches, castor oil is utilized for the treatment of blepharitis and dry eye problems[9], as a laxative for constipation treatment, and in various dermatological conditions owing to its wound-healing properties. It is also used to treat baldness, dermatitis, and regeneration in burn patients[10].

This study aimed to determine the efficacy of castor oil in preventing stricture development in rats by using an experimental CEB model. In this study, tissue hydroxyproline levels, TNF-alpha and VEGF levels for tissue damage and inflammation, and Caspase-3 for apoptosis pathway-associated tissue damage were measured to evaluate wound healing. In addition to standard histopathological techniques, tissue damage, oxidative stress, and response to treatment were assessed for the first time by measuring neopterin, sST2, IL-6, and IL-33 proteins. Neopterin is a marker of oxidative stress induced by cellular immune response. Elevated levels of neopterin have been observed in viral infections, intracellular pathogens, autoimmune diseases, allograft rejection, trauma, burns, and malignant diseases[11]. ST-2 (Suppression of Tumorigenicity 2 protein) is a protein belonging to the IL-1 family that exists in both soluble and transmembrane forms. The ligand for ST-2 is IL-33. ST-2 serves as an indicator of tissue damage in sepsis, is significant for mortality from trauma, serves as an important marker of heart failure, and is used in the prognosis of chronic kidney failure[12].

Results

Laboratory process

The laboratory process was completed by two researchers, who used the same laboratory clothes and equipment. At the end of the study, the number of subjects in each group was: G1 $n=8$, G2 $n=8$, G3 $n=8$, G4 $n=6$, and G5 $n=8$, with a total of 38 subjects completing the process. The sham group appeared to be the healthiest, whereas the G4 group appeared to be the least healthy. Although no complications were observed during the gavage procedure in G5, castor oil administration was paused for one day due to the presence of loose stools in two subjects at two different times.

Results of subjects' weight measurements and surgery duration

The weights of the groups before and after decapitation (day 21) were not significantly different from the average weights of the groups before modeling ($p=0.060$); a significant difference was found before decapitation ($p<0.029$). In G5, the average weight before decapitation was significantly higher than that before modeling ($P=0.003$).

In all study groups, there was no significant difference between the esophageal lengths measured during and at the end of the study ($p>0.05$). No statistically significant difference was found between the surgical durations of the groups ($p=0.060$).

Biochemical evaluation

The changes in IL-6 (pg/mL), IL-33 (ng/L), ST2/IL-1 (ng/mL), TNF-alpha (ng/L), neopterin (nmol/L), and HYP (µg/g protein) levels measured in this study are presented in Table 1. Before comparing the groups, we investigated whether there was a covariate effect on independent variables in the statistical analysis of the weights of the rats distributed to the groups based on randomization. There was no covariate effect of weight on any independent variable ($p>0.05$).

The mean IL-6 levels were not significantly different between the groups ($P=0.220$). There was also no significant difference in IL-33 levels between the groups ($p=0.051$). TNF-alpha and ST2/IL-1 levels were not significantly different between groups ($p=0.295$ and $p=0.850$, respectively) Although not statistically significant, the general pattern showed a gradual increasing trend for neopterin and IL-33 in the application and treatment models (G4-5). (Fig. 1).

There was a significant difference in neopterin levels between groups ($p<0.001$) (Fig. 1). Although there was no significant difference between G4 and G5 ($p>0.05$); both of these groups had significantly higher neopterin levels compared to the other groups ($p<0.05$). The Neopterin level in G1 was lower than that in all groups, except G3 ($p<0.05$). There was also a significant difference in the HYP levels between the groups ($p=0.016$). The HYP level in G5 was significantly higher than that in G1 ($p<0.05$) (Fig. 2).

		n	Mean.	SS	Median[IQR]	Rank Mean.	*p*
IL-6 (pg/mL)	Sham	8	37.18	16.34			0.220*
	Control	8	44.37	9.83			
	Early Stage	8	38.32	9.17			
	Late Stage	6	31.39	13.57			
	Oral Application	8	28.65	18.21			
IL-33 (ng/L)	Sham	8	15.43	10.35			0.051*
	Control	8	19.49	10.71			
	Early Stage	8	18.85	11.19			
	Late Stage	6	31.38	9.65			
	Oral Application	8	25.92	9.32			
TNF-Alfa (ng/L)	Sham	8	141.72	61.50			0.295*
	Control	8	115.00	52.01			
	Early Stage	8	87.14	50.48			
	Late Stage	6	146.51	72.92			
	Oral Application	8	130.43	55.47			
ST2/IL-1 (ng/mL)	Sham	8	0.98	0.59	1.1[0.9]	18.75	0.850**
	Control	8	1.23	0.35	1.2[0.5]	23.00	
	Early Stage	8	0.99	0.32	1.0[0.5]	16.63	
	Late Stage	6	1.26	1.14	1.1[2.2]	19.50	
	Oral Application	8	1.12	0.38	1.1[0.7]	19.63	
Neopterin (nmol/L)	Sham	8	0.71^{b}	0.20	0.7[0.4]	9.00^{d}	**< 0.001****
	Control	8	1.33^{a}	0.42	1.2[0.7]	21.56^{bc}	
	Early Stage	8	1.59^{ab}	2.58	0.7[0.5]	11.88^{cd}	
	Late Stage	6	2.59^{a}	0.87	2.7[1.6]	31.67^{a}	
	Oral Application	8	1.90^{a}	0.82	1.9[1.3]	26.44^{ab}	
HYP (ug/gr prot)	Sham	8	581.45	102.08	584.9[145.2]	10.13^{b}	**0.016****
	Control	8	1063.31	358.90	926.0[639.2]	25.63^{a}	
	Early Stage	8	864.12	505.95	723.4[938.1]	17.25^{ab}	
	Late Stage	6	862.01	672.15	694.4[649.0]	17.17^{ab}	
	Oral Application	8	1288.87	579.14	1305.6[1178.1]	26.75^{a}	

Table 1. Variation in biochemical parameters according to groups. *One-way ANOVA; **Kruskal-Wallis test. There was a significant difference between groups with different letters ($p < 0.05$).

Histopathological evaluation

Submucosal and tunica muscularis fibrosis, muscularis mucosa damage

The presence of submucosal fibrosis (SF), muscularis mucosa damage (MMD), and tunica muscularis fibrosis (TMF) in the groups were compared and the results are presented in Table 2. (Fig. 3.). The presence of SF varied significantly between the groups ($p < 0.001$). Although SF was absent in G1, it was present in both G2 and G3 groups. In G4, SF was observed in 66,7% of cases and in G5, it was observed in 50,0% of cases.

The MMD showed significant differences among the groups ($p < 0.001$). MMD was absent in G1 and G4. However, it was present in all G3 cases. The incidence of MMD in G2 and G5 was 25.0% and 12.5%, respectively.

The presence of TMF varied significantly among the groups ($p < 0.025$). TMF was not observed in the G1 phase. The group with the highest incidence of TMF was G2 (62.5%).

Total stenosis score, VEGF, caspase-3 ratios

The total stenosis score (TSS), VEGF, and caspase-3 ratios of the groups were compared (Figs. 4 and 5). TSS showed significant variation among the groups ($p < 0.001$). The lowest TSS was observed in G1 (median [IQR]: 0.0[0.0]) and the highest in G3 (median [IQR]:2.0[1.0]); this difference was statistically significant ($p < 0.05$). Both G4 and G5 had significantly lower TSS concentrations than G2 and G3 ($P < 0.05$). G2 (median [IQR]:2.0[1.5]) did not differ significantly from G3 ($p > 0.05$) but had a higher TSS than G1, G4, and G5 ($p < 0.05$).

The VEGF ratio showed a significant variation among the groups ($p = 0.001$). The lowest VEGF ratio was observed in G1 (median [IQR]:1.0[1.0]) and the highest in G3 (median [IQR]:3.0[0.0]); this difference was statistically significant ($p < 0.05$). There was no significant difference in the VEGF ratio between the sham and control groups (median [IQR]:2.0[0.0]) or the Oral Application group (median [IQR]:2.0[0.8]) ($p > 0.05$).

There was also a significant difference in the caspase-3 ratio between groups ($p = 0.005$). The highest caspase-3 ratio was observed in G2 (median [IQR]:2.0[0.75]), which did not differ significantly from that in G4 (median [IQR]:1.5[1.0]) ($p > 0.05$). However, G2 had a significantly higher caspase-3 ratio than G1 (median [IQR]:1.0[0.0]), G3 (median [IQR]:1.0[0.0]), and G5 (median [IQR]:1.0[0.0]) ($p < 0.05$).

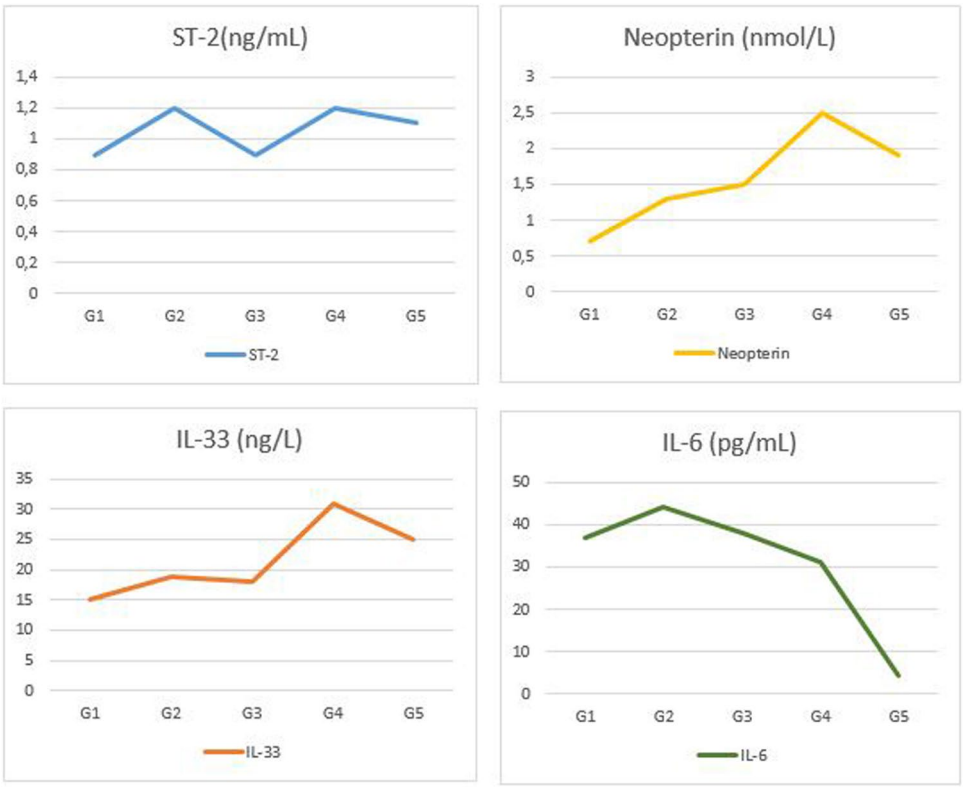

Figure 1. Change curves of serum ST-2, neopterin, IL-33, and IL-6 values among the groups.

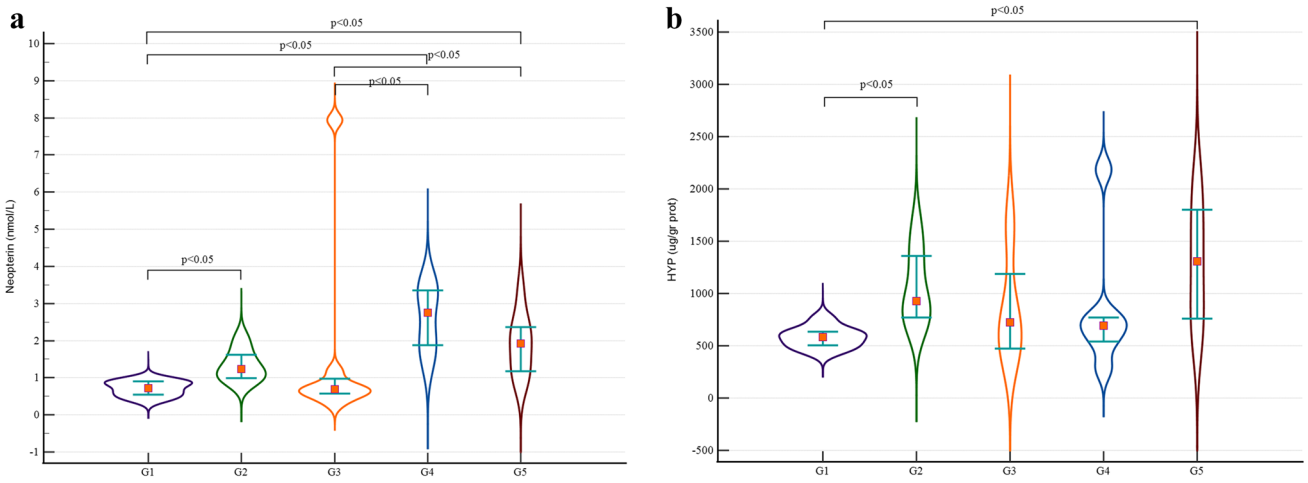

Figure 2. **a** Violin plot illustrating the levels of Neopterin (nmol/L) in groups. The red dot represents the median, and the green bars represent the interquartile range (25th–75th quartiles). **b** Violin plot illustrating the levels of HYP (ug/gr prot) in groups. The red dot represents the median, and the green bars represent the interquartile range (25th–75th quartiles).

Histopathological damage score

The histopathological damage scores (HDS) of the groups were compared, and a statistically significant difference was found between the groups ($p < 0.001$). The lowest HDS was observed in G1(median [IQR]:0.0[0.0]), and the highest in G2 (median [IQR]:4.0[1.0]); this difference was statistically significant ($p < 0.05$). There was no significant difference between the G2 and G3 groups (median [IQR]: 3.0 [2.0]) ($p > 0.05$), and both groups had significantly higher HDS than G1, G4, and G5 ($p < 0.05$).

		Sham		Control		Early Stage		Late Stage		Oral Application		p*
		n	%	n	%	n	%	n	%	n	%	
Submucosal Fibrosis	No	8	100.0	0	0.0	0	0.0	2	33.3	4	50.0	**<0.001**
	Yes	0	0.0	8	100.0	8	100.0	4	66.7	4	50.0	
Muscularis Mucosa Damage	No	8	100.0	6	75.0	0	0.0	6	100.0	7	87.5	**<0.001**
	Yes	0	0.0	2	25.0	8	100.0	0	0.0	1	12.5	
Tunica Muscularis Fibrosis	No	8	100.0	3	37.5	5	62.5	5	83.3	4	50.0	**0.025**
	Yes	0	0.0	5	62.5	3	37.5	1	16.7	4	50.0	

Table 2. Comparison of the presence of SF, MMD, and TMF among groups *Likelihood ratio chi-square value

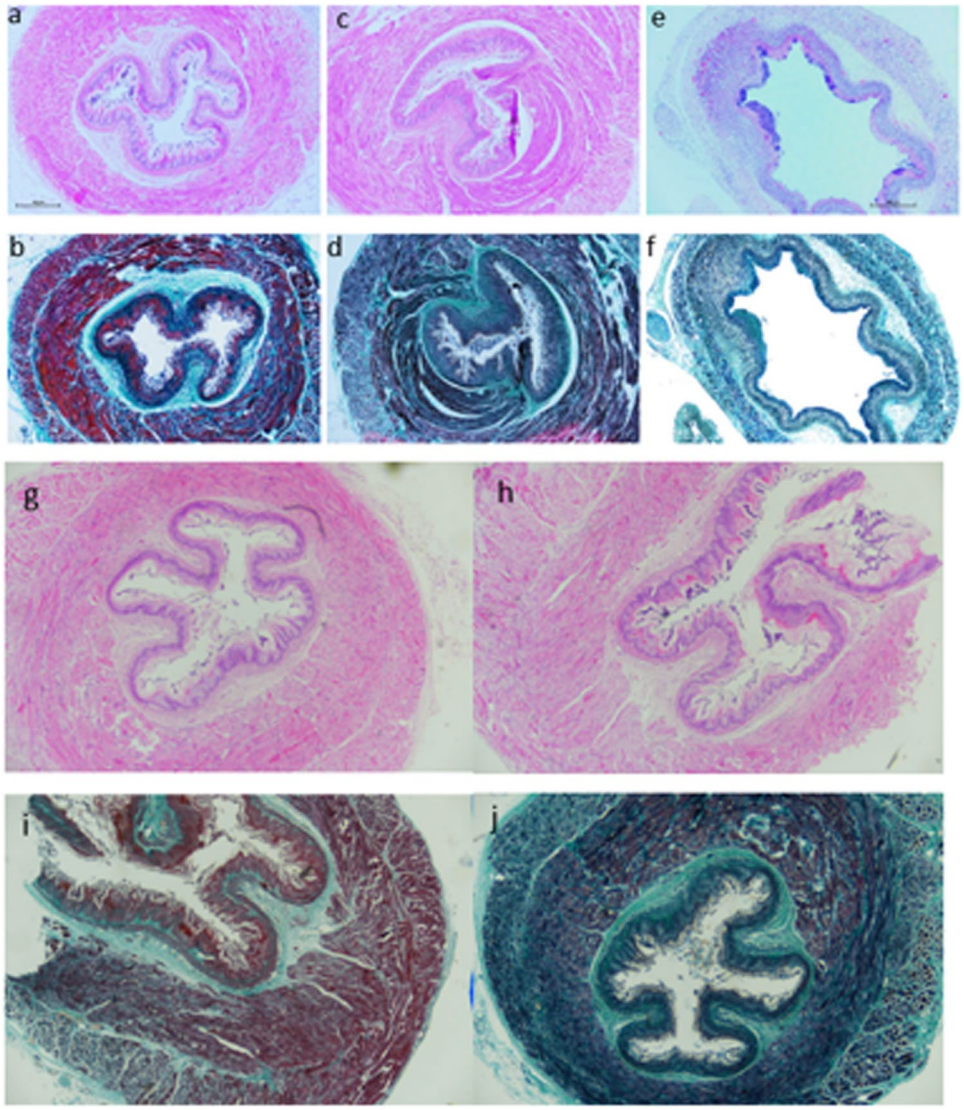

Figure 3. Histological findings of the esophagus in H&E and Masson's trichrome staining. **a**, **b** No histological damage was observed in G1. **c**, **d** G2, narrowing of the esophageal lumen, submucosal edematous changes, and collagen deposition in the submucosa and muscularis mucosa were observed. **e**, **f**, G3, widespread ulceration in the mucosal epithelium, submucosal suppurative inflammation, and edematous changes were observed. **g**, **h**, mild stricture was observed in the lumen. While there was a slight increase in submucosal collagen, no muscularis propria damage was observed in G4. **f**, **i**, submucosal and muscularis mucosal collagen deposition observed in G5 (Hematoxylin& Eosin section, original magnification, x40).

Discussion

CEB have high mortality and morbidity in the acute phase and become chronic in the long term if an esophageal stricture is present. CEB is one of the most common causes of upper gastrointestinal stenosis in children[13].

In this study, we aimed to investigate the effectiveness of castor oil in rats with corrosive esophageal burns in preventing the formation of esophageal stenosis, which is generally highly resistant and requires long-term treatment in children under 5 years of age.

Esophageal strictures in the first three weeks can cause nutritional defects due to feeding difficulties, swallowing difficulties, and loss of appetite. In our study, we observed statistically significant weight gain in the group that received castor oil for 21 days (G5). We believe that this result is primarily due to the inhibitory effect of castor oil on esophageal stenosis; however, it is also known that castor oil has nourishing effects. Additionally, we did not observe any weight gain in G4. We believe that this result could be due to the single-dose castor oil application and could be due to different reasons (weaker subjects in randomization) in G4. Akay et al. stated that St. John's wort oil had effects on nutrition and weight gain was observed in the subjects in the prevention of stricture[14].

In the CEB model, the esophageal tissue was damaged by NaOH. In the acute necrotic phase, hemorrhage and thrombosis develop within the first three days, followed by local necrosis and angiogenesis. In this phase,

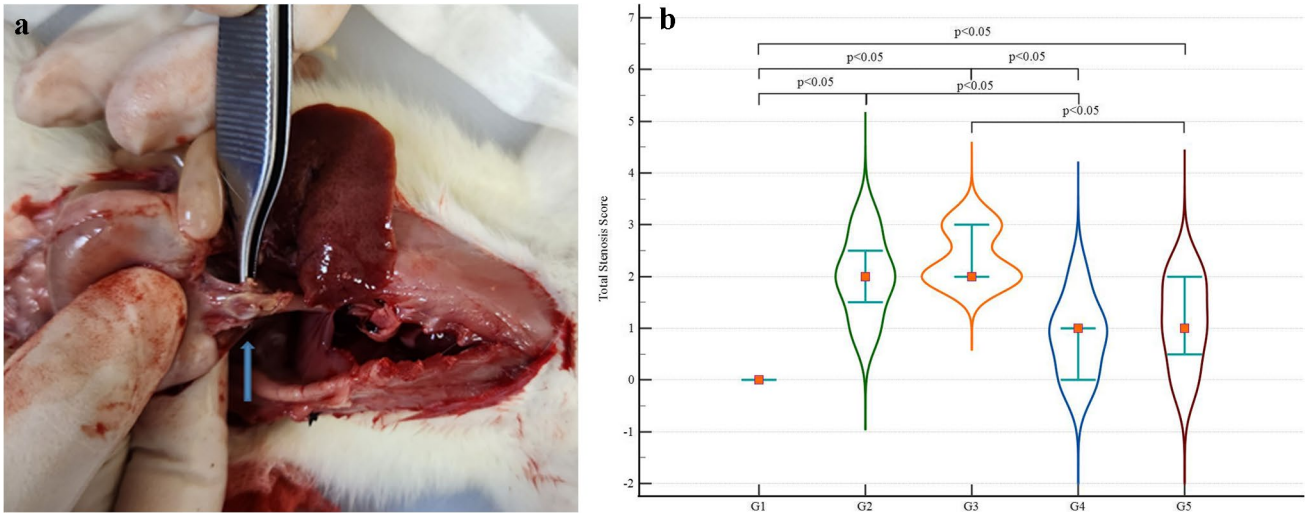

Figure 4. **a** Appearance of the damaged esophageal segment. **B** Violin plot showing the groups' total stenosis score: G1: Sham, G2: Control, G3: Early Stage, G4: Late Stage, G5: Oral. The red points show the median. Green bars show the IQR (25–75 percentiles).

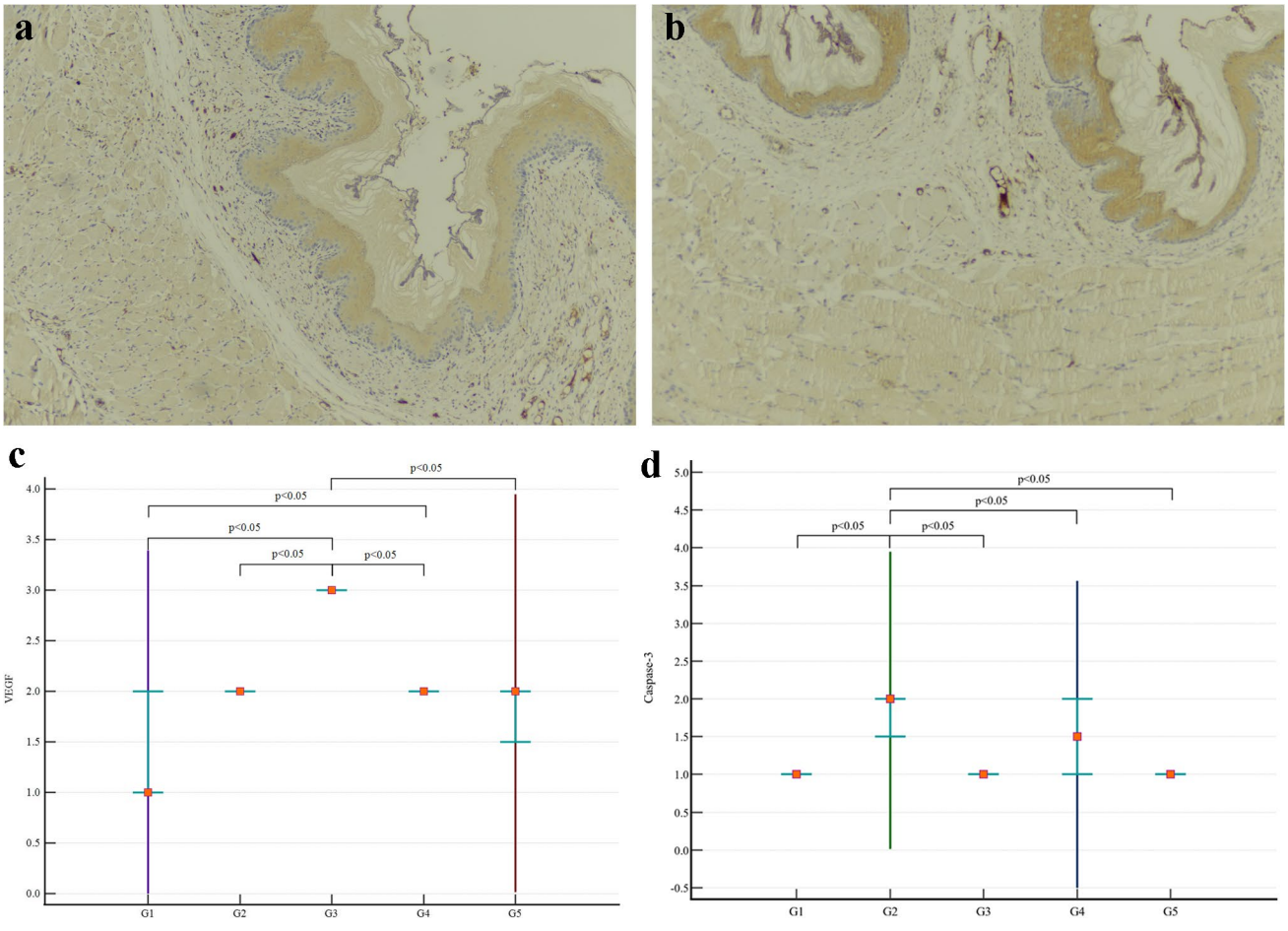

Figure 5. **a** Immunohistochemically Caspase-3 nuclear staining in the submucosal tissue in Group 2. **b** Immunohistochemically, VEGF staining of the vascular structures in Group 4. (Immunoperoxidase, original magnification, x200). **c** Violin plot showing the groups' VEGF: G1: Sham, G2: Control, G3: Early Stage, G4: Late Stage, G5: Oral. The red points show the median. Green bars show the IQR (25–75 percentiles). **d** Violin plot showing the groups' Caspase-3 rate: G1: Sham, G2: Control, G3: Early Stage, G4: Late Stage, G5: Oral. The red points show the median. Green bars show the IQR (25–75 percentiles).

inflammation is expected to be triggered in the serum, oxidative stress markers to increase, necrosis to begin, and activation of apoptotic pathways[3]. In our study, we used G3 to evaluate the early phases. In the G3 data, weight loss started, the esophageal length shortened, and IL-6, which only responds to the acute phase, increased differently; the tissue damage markers ST2/IL-1 and TNF-alpha decreased, while the oxidative stress and early inflammatory response marker neopterin started to rise. In the immunohistochemical data, an increase in the VEGF ratio and a decrease in caspase-3 protein related to apoptotic pathways were observed in G3. These data show that even a single dose of topically applied castor oil was effective.

Type 1 immunity consists of T helper 1(+) IFN-γ-producing group 1 ILCs, CD8 cytotoxic T cells (TC1), and CD4(+) TH1 cells, which protect against intracellular microbes through activation of mononuclear phagocytes. Type 2 immunity consists of GATA-3(+) ILC2s, TC2 cells, and TH2 cells producing IL-4, IL-5, and IL-13, IL-33 which induce mast cell, basophil, and eosinophil activation, as well as IgE antibody production, thus protecting against helminthes and venoms[15,16]. IL-33 is a member of the IL-1 family and is found in endothelial cells, epithelial cells, and fibroblasts. IL-33 plays a role in neo-angiogenesis and in the response to cellular damage or stress.

IL-33 enhances the levels of ST2 and FOXP3, boosts Treg cell functions, reduces colon tissue damage severity, and helps to prevent the initiation of intestinal inflammation[17]. As a multifaceted cytokine, IL-33 not only supports Type 2 inflammatory processes but also promotes immune regulation by expanding Foxp3 + Tregs[18]. Additionally, IL-33 has been shown to influence macrophages with alternatively activated macrophage-like traits, which become predominant in the later stages of both infectious and sterile inflammation, aiding in inflammation resolution, improvement of Type 1 inflammatory responses and adaptive immunity, and the regulation and promotion of Type 2 immune responses and repair processes[19]. The ST2 protein is effective in triggering tissue damage, inflammation, and remodeling. It plays a significant role in inflammation, heart disease, and tumor development[13]. ST2 is thought to limit IL-33 bioavailability by acting as a decoy receptor and is increased in patients with active inflammatory bowel disease suggesting that the chronic inflammatory tissue environment may antagonize IL-33 activity[17].

In our study, as shown in the graphs in Fig. 1, although not statistically significant, there is a notable trend of increased IL-33 levels in the treatment groups, while ST2 levels remained unchanged. We would like to highlight that this increase in IL-33 may be due to its activities in preventing gastrointestinal mucosal tissue damage and intestinal inflammation, as observed in the studies by Schiering C. and colleagues.

Trauma and burns cause type 1 immunity responses, T cell-macrophage interactions and increase neopterin levels[20]. Neopterin were measured for the first time in this study using the CEB model.

We know that neopterin is produced as a metabolite of Type 1 immune responses, triggered by interferon-alpha released from TH1 lymphocytes, in conjunction with TNF-alpha and oxygen radicals. This supports the observation that the serum concentration curves of TNF-alpha in our study should resemble those of neopterin. However, unlike the caspase-3 measurements, which assess cell death, an opposing trend is observed. In contrast, our study shows a significant increase in neopterin levels in the treatment groups. This might be attributed to neopterin's tendency to increase even in emotional states such as stress or depression[21]. It is considered that the daily gavage treatment of Group 5 subjects and the fact that Group 4 subjects generally exhibited the lowest overall well-being after randomization may have contributed to this effect.

In many studies of esophageal stricture, tissue HYP levels have been evaluated in terms of stricture formation[22]. Although low tissue HYP levels were considered positive in response to stricture treatment, elevated levels were also observed in the treatment groups as in the literature and same as our study[12]. In this study, looking at the HYP levels, we observed that the oral application group (G5) had an elevated HYP level close to that of the control group. We thought that this increase could be secondary to esophageal mucosal damage caused by the daily gavage procedure, even though we tried to administer it carefully. In addition, when pathological evaluation was performed, the fibrosis rate in G5 was 50%, and the TSS was 1.

Evaluation of esophageal tissue in pathological assessments is important. Effectiveness was assessed based on HDS, TSS, SF, MMD, and TMF. In this study, the esophagus was significantly better in G4 and G5 than in G2. The HDS was 4 in G2 and 1.5 in G5, and a statistically significant decrease in TSS was found in G4 and G5. These results support the anti-sclerotic and wound healing effects of castor oil.

In pathological angiogenesis, VEGF facilitates the mobilization of inflammatory cells to damaged areas, inflammatory processes, and induction of pro-angiogenic factor synthesis[23]. Hypoxia is the most important factor that triggers angiogenesis; however, other factors such as hypoglycemia, hypertension, low pH, mechanical stress, and chronic inflammation also trigger angiogenesis. Vascular response is the primary response mechanism in wound healing. At this stage, neo-angiogenesis begins and is completed within the first three days[24]. In this study, VEGF and caspase-3 were measured on biopsy samples obtained from the CEB area. The VEGF ratio was the lowest in G1 and the highest in G3, and this difference was statistically significant. An increase in the VEGF ratio during the first three days of the vascular response phase was observed as an appropriate result in the wound healing response. However, no significant differences were observed among G2, G4, and G5. This result was thought to be secondary to the inflammatory process triggering VEGF synthesis, which is also observed in tissue healing[25].

Caspase-3 is responsible for the fragmentation and death of the target molecules[24]. In this study, the highest caspase-3 ratio was observed in G2, which was significantly higher than that in G5. This result demonstrates that oral application of castor oil supports remodeling.

This is the first study to evaluate the efficacy of castor oil in an experimental CEB model and to use inflammatory proteins, such as ST-2/IL-33 and neopterin. Additionally, it includes a comprehensive project that incorporates immunohistochemical studies of VEGF and caspase-3 along with biochemical data. The results of this study showed that castor oil, consistent with literature data, is effective in tissue healing of esophageal corrosive burns and can be a preventive treatment alternative in the development of strictures. However, we

believe that there are some limitations due to the experimental nature of the study and the limited number of subjects.

Method

Animals, study design and 21-day follow-up period

This study was conducted at our university's animal experimentation laboratory, with approval from the local ethics committee for animal experiments (2023/15). The researchers completed the study in accordance with the ARRIVE guidelines.

Fifty Wistar-Albino rats were divided into 5 groups and an experimental CEB model were created by using the Gehanno and Guedon method[26].

Group 1- Sham ($n=10$): Instead of 1 ml corrosive 37,5% NaOH solution, 1 ml saline was used for modelling.
Group 2– Control ($n=10$): CEB modelling was performed.
Group 3- Early Stage Topical Application ($n=10$): After modelling and washing with saline, 1 ml. castor oil given for 90 s, without aspiration. Postoperative 3rd day decapitation was performed.
Group 4- Late Stage Topical Application ($n=10$): After modelling and washing with saline, 1 ml. castor oil given for 90 s, without aspiration. Postoperative 21th day decapitation was performed.
Group 5- Oral Application ($n=10$): On the first postoperative day, 1 ml castor oil was administered by oral gavage. The same dose was administered daily for 21 days. The procedures were paused in animals with diarrhea. Postoperative 21th day decapitation was performed.

Corrosive esophageal burn injury modelling

After 12 h of fasting, rats were randomly divided into five groups. The rats were anesthetized with 100 mg/kg ketamine and 15 mg/kg 2% xylazine HCI. After anesthesia, the subjects' weights were measured, and their abdomens were shaved. Fixed with drape on a 30-degree table with reverse Trendelenburg position, a 3 cm midline abdominal incision was made under sterile surgical conditions. The stomach was gently pulled, and the esophagus was reached. A 5 Fr orogastric catheter was advanced through the proximal esophagus and a 24 G cannula was placed from the stomach to the distal esophagus. A 2 cm esophageal segment was tied with 3/0 silk sutures over the proximal and distal catheters to prevent leakage. One milliliter of a 37.5% NaOH solution was added to the segment for 90 s. The fluid was then aspirated. After washing with saline, the silk sutures and cannulas were removed and the abdomen was sutured with a 4/0 atraumatic silk suture. In G3 and G4, 1 mL was washed with saline. castor oil for 90 s without aspiration, and the silk sutures and cannulas were removed. To prevent dehydration during the early stages, 10 ml of 0.09% saline was injected into the intraperitoneal cavity.

All rats were fed a liquid diet on the first postoperative day and pellets were provided ad libitum on the second day. All rats were weighed on the day of modelling before decapitation.

Histopathological evaluation

After decapitation, the distal esophagus of 50 rats was excised and fixed in a 10% neutral formalin solution for 24 h. Subsequently, the materials were embedded in paraffin blocks, and sections of four micrometers thickness were obtained and stained with Hematoxylin & Eosin and Masson's trichrome histochemical stains. A histopathological examination under light microscopy was conducted, and the stenosis index was assessed to evaluate the degree of inflammation and fibroblast proliferation. When determining the stenosis index, an increase in the amount of submucosal collagen was scored on a scale of 0 to 5 in three categories. These three categories include damage to the muscularis mucosa, tunica muscularis, and collagen deposition.

Subsequently, to evaluate the changes in tissue damage before and after treatment, sections were stained with caspase 3 and VEGF immunohistochemically stains, and assessment was performed based on staining intensity in four categories within a high-magnification field. These categories were evaluated as follows: staining intensity 0–25%: 1; staining intensity 25–50%: 2; staining intensity 50–75%: 3; and staining intensity 75–100%: 4. The staining intensity and stenosis index in these categories were statistically evaluated.

The total stenosis score (TSS) was determined by measuring the esophageal wall thickness at its thickest point and the lumen diameter at its widest point across all tissue sections (TSS = wall thickness/lumen diameter)[27].

The histopathological damage score (HDS) for caustic esophageal burn injury was evaluated semi-quantitatively using esophageal collagen deposition and damage (Table 3).

	Criteria of injury	Score
Increase in submucosal collagen	None	0
	Mild (submucosal collagen at least twice the thickness of the muscularis mucosa)	1+
	Marked (submucosal collagen more than twice the thickness of the muscularis mucosa)	2+
Muscularis mucosa damage	None	0
	Present	1+
Damage with collagen deposition in tunica muscularis	None	0
	Mild (collagen deposition around the smooth muscle fibers)	1+
	Marked (same as mild, with collagen deposition replacing some muscle fibers)	2+

Table 3. The histopathological damage score for caustic esophageal burn criteria of injury score.

Biochemical evaluation

Blood samples were obtained from rats via intracardiac puncture and then sacrificed. The collected blood samples were placed in biochemical tubes without anticoagulants. After waiting for 30 min for blood clotting, the samples were centrifuged at 1800 × g for 10 min, and the obtained serum samples were stored at -80 °C in a deep freezer until analysis. Following the collection of blood samples from the rats, esophageal tissue was excised, washed with isotonic sodium chloride (NaCl) solution, and then homogenized in a 0.1 M phosphate buffer solution containing 0.1mM EDTA at a ratio of 1/4 (weight/volume) and pH 7.0. The homogenized material was then centrifuged at 14,000 rpm for 20 min at 4 °C, and the supernatant was separated and stored at -80 °C.

Measurement of serum TNF-alpha, IL-6, ST-2, IL-33, neopterin, and tissue hydroxyproline levels: Measurements were performed using commercially available ELISA kits. In this method, measurements were performed using a sandwich ELISA. Each well of the microplate provided in the commercial kit was coated with specific antibodies. Standards and collected blood samples were added to plate wells and incubated at 37 °C for 90 min. Biotin-labeled antibodies were added and incubated at 37 °C for 1 h, followed by aspiration and washing. Biotinylated Avidin-Horseradish Peroxidase (HRP) conjugate was added to each well, incubated at 37 °C for 30 min, aspirated again, and washed five times. A surfactant component was added to each well, and after incubation at 37 °C for 15 min, the reaction was stopped by adding sulfuric acid solution, resulting in the formation of a yellow color in the wells. The absorbance was measured at an optical density of 450 nm, and a standard curve was drawn to calculate the concentrations of the unknown samples.

Protein measurement of the esophageal tissue was conducted using a colorimetric method, and the levels of tissue hydroxyproline were reported as mg/L of tissue protein.

Statistical analysis

A 5% statistical significance level was considered in the calculations and interpretations, and two-tailed p-values are presented. Before parametric tests, normal distribution control was performed with the Shapiro-Wilk test and homogeneity control of group variances was performed with the Levene's test. As a descriptive statistic; mean and standard deviation (SD) were calculated for continuous variables, median and interquartile range (IQR) for score variables, and frequencies (n, %) were calculated for categorical variables. Paired t-test or Wilcoxon signed-rank test was used to compare dependent groups. To compare independent groups, one-way ANOVA, Welch's ANOVA, or Kruskal-Wallis test was used, depending on the situation. As a multiple comparison test, the Tukey-Kramer test was used after ANOVA, the Games-Howell test was used after Welch ANOVA, and the Dunn test was used after the Kruskal-Wallis test. The multiple comparison tests were performed at $\alpha = 0.05$, and the results were represented with letters. Chi-square test was used in the analysis of categorical variables. In the chi-square test, the Likelihood ratio test statistic was calculated instead of Pearson's since the assumptions were not met. SPSS v28 (IBM Corp.; Armonk, NY, USA) and MedCalc v22.005 (MedCalc Software Ltd, Ostend, Belgium) statistical software were used in the calculations.

Financial disclosure and conflict of interest

This research was approved by the Local Ethics Committee at our university and adhered to the principles outlined in the Guide for the Care and Use of Laboratory Animals (U.S.). National Institute of Health Publication # 85 – 23, revised 1996). The authors affirmed that the study was conducted in accordance with the Declaration of Helsinki. The authors declare that they have no conflicts of interest or financial agreements with pharmaceutical or biomedical companies relevant to the subject matter discussed in this manuscript. Funding for this study was provided by the Scientific Research Fund of TUBITAK (The Scientific and Research Council of Türkiye), project number 123S428, ensuring no plagiarism or content duplication. M.D. Imge Benzes worked on this project as a scholarship student.

Data availability

Detailed data of this study can be seen by searching the TUBITAK project database module with the project code numbered 123S28. Since the project is in the publication process, the data cannot yet be seen on the internet, but it will be visible on the TUBITAK platform after its publication (https://search.trdizin.gov.tr/tr/proje/ara?=&searchName=&order=year-DESC&page=1&limit=20). It is available in the Ordu University Scientific Research Fund repository (https://ebap.odu.edu.tr/?act=guest&act2=projeler&durum=tamam). With this the datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

A.K.A and T.N. conceived of the presented idea. A.K.A developed the theory. Y.K.A. verified the analytical methods and statistical analysis. A.K.A., O.Y., C.Y.G. carried out the experimental model. O.K.K. performed histopathological section of the study. A.B.G. and T.N. performed biochemical section of the study. A.K.A. wrote the manuscript with support from C.Y.G., O.K.K., A.B.G, Y.K.A. All authors discussed the results and contributed to the final manuscript. All authors discussed the results and commented on the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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