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Protective Effects of Dexpanthenol and Y-27632 on Stricture Formation in a Rat Model of Caustic Esophageal Injury

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Background. **This experimental study was conducted to investigate the effect of dexpanthenol (converted in the body to pantothenic acid) and Y-27632 (a selective Rho-kinase inhibitor) on stricture formation after caustic (alkaline) esophageal injury in rats.**

Materials and Methods. **Sixty male Wistar albino rats were randomly allocated into six groups. In group 1 (sham) the distal esophagus was isolated and cannulated but no caustic injury was induced. In all remaining groups, a caustic esophageal burn was induced with 50% sodium hydroxide solution for 90 s and drug treatment was given by daily intraperitoneal injection, beginning 24 h after injury and continuing for 21 d. In group 2 (controls), animals were treated with 0.9% saline; in groups 3 and 4, with 50 and 500 mg/kg/d of dexpanthenol, respectively; and in groups 5 and 6, with 0.3 and 3 mg/kg/d of Y-27632, respectively. Rats were sacrificed 22 d after caustic injury and the distal esophagus was isolated for histopathology and biochemical investigation.**

Results. **Stenosis index and collagen deposition scores were significantly lower in both the dexpanthenol and Y-27632 treated groups ($P < 0.05$). Dexpanthenol and Y-27632 treatment markedly depressed esophageal tissue malondialdehyde and hydroxyproline levels.**

Conclusion. **In this experimental model of caustic esophageal stricture, dexpanthenol and Y-27632 significantly attenuated esophageal stricture formation. These findings indicate that inhibition of Rho-kinase or dexpanthenol administration may offer novel therapeutic approaches in the treatment of caustic esophageal injury.**

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Key Words: **caustic esophageal injury; dexpanthenol; Y-27632.**

INTRODUCTION

Caustic esophageal injury continues to be a major health problem in childhood in both developed and developing countries [1–3]. Caustic injury can lead to potentially fatal sequelae such as esophageal perforation, aspiration pneumonia, or esophageal cancer. As the chemical burn heals, an esophageal stricture may develop, requiring multiple dilatations or even esophageal replacement [1–4]. The optimal approach to minimize esophageal damage after caustic ingestion is controversial. Various treatment protocols have been suggested. Corticosteroids, antibiotics, proton pump inhibitors, and H_2 receptor blockers are widely used in an effort to prevent stricture formation [1, 4, 5]. Ongoing experimental research continues to attempt to identify novel treatment strategies [6–10].

Dexpanthenol is an alcoholic analogue of pantothenic acid, a member of the B complex vitamins (vitamin B5). It is enzymatically oxidized to pantothenic acid, which is widely distributed in tissues as coenzyme A [11]. Dexpanthenol is used in clinical practice for its beneficial effects on wound healing [11–13].

Rho-kinase is an enzyme that plays a critical role in various cellular functions, such as changes in cell morphology, cell motility, cross-talk between actin stress fibers, and cytokine production [14, 15]. These functions are integral to contraction, migration, and proliferation of constituent cells, particularly in the gut. Rho- and Rho-kinase-mediated signaling pathways are important in smooth muscle contraction and migration [16, 17]. *In vitro* studies have shown that

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Rho-kinase is also involved in wound repair [18]. Y-27632 is a selective Rho-kinase inhibitor that acts by competing with the enzyme's ATP binding site [19]; this agent can suppress the formation of actin stress fibers and stimulate wound closure in bronchial epithelial cells [20, 21]. The role of Rho-kinase in healing esophageal wounds and the potential underlying mechanisms are uncertain. No biochemical or histopathologic studies of corrosive esophageal burns treated with dexpanthenol or a Rho-kinase inhibitor have yet been reported. We conducted an experimental study to investigate the effect of dexpanthenol and Y-27632 on stricture formation in a rat model of caustic (alkaline) esophageal injury.

MATERIALS AND METHODS

Experimental Animals

The study was conducted using adult Wistar albino rats housed at 20–24°C with 12 h light and 12 h dark cycles and supplied with standard rat chow and water *ad libitum*. The study conformed to the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health Publication no. 85-23, revised 1996). The experimental protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Gaziantep.

Caustic Esophageal Injury

A caustic esophageal injury was produced using the model described by Gehanno and Guedon and modified by Liu and Richardson [22, 23]. After a 12 h fast, animals were anesthetized with 15 mg/kg 2% xylazine (Rompun; Bayer, Istanbul, Turkey) plus 100 mg/kg ketamine hydrochloride (Ketalar; Pfizer, Istanbul, Turkey) given subcutaneously. A midline laparotomy was performed and a 2 cm long segment of abdominal esophagus mobilized. A 5F feeding catheter was introduced orally into the upper part of this esophageal segment. A 14G intravenous catheter was introduced through the stomach wall into the distal part of the isolated esophageal segment for drainage, and the ends of the isolated 2 cm segment were tied externally with a 3/0 silk ligature. A 50% concentrated solution of sodium hydroxide was infused at a pressure of 30 cm water through the proximal catheter for 90 s. The esophageal segment was then immediately irrigated with 20 mL distilled water for 60 s, after which the catheters were withdrawn, the silk ligatures removed, and the laparotomy incision closed. The animals then received 10 mL 0.9% saline *via* subcutaneous injection and were allowed to feed *ad libitum* from 12 h postoperatively. Treatment protocols were commenced 24 h after the caustic esophageal burn [7] and continued for 21 d.

Study Design

Sixty male rats were randomly allocated into six equal groups. In group 1 (sham group) all steps of the operative procedure were performed, but the isolated esophageal segment was infused with 0.9% saline only and the rats received daily intraperitoneal injections of isotonic saline postoperatively. In all remaining groups a caustic esophageal injury was induced but a different agent was used for the postoperative regimen of daily intraperitoneal injections: in group 2 (control) this consisted of 0.9% saline; in groups 3 and 4, the rats were injected with 50 mg/kg/d and 500 mg/kg/d of dexpanthenol (Bepanthene; Bayer, Istanbul, Turkey), respectively; in groups 5 and 6, the animals were injected with 0.3 mg/kg/d and 3 mg/kg/d of Y-27632 (Tocris Cookson Ltd. Bristol, UK), respectively. Twenty-two days after caustic injury, the animals were sacrificed under anesthesia by thoracotomy and cardiac incision and the distal esophagus was harvested, fixed in 10% buffered formalin, and embedded in paraffin. A sample of each specimen was stored in individual sealed containers at –40°C for subsequent measurement of tissue malondialdehyde (MDA) and hydroxyproline concentrations.

Histopathology

Transverse sections of the esophageal specimens (4–5 μm) were obtained and stained with hematoxylin and eosin (H and E) and Masson's trichrome. Histopathologic sections were examined by a single pathologist blinded to the treatment group. A stenosis index (wall thickness/lumen diameter) was calculated in each animal [24], and mean values of this index in each group were compared statistically. Collagen accumulation in the submucosa and muscular layer was assessed and scored semiquantitatively (Table 1) [24].

Biochemical Analyses

MDA Determination

Esophageal tissue concentrations of MDA were determined by Wasowicz's method based on the reaction of MDA with thiobarbituric acid at 95–100°C [25]. Fluorescence intensity was measured in the upper n-butanol phase by fluorescence spectrophotometry (model F-4010; Hitachi, Tokyo, Japan) adjusted to an excitation at 525 nm and emission at 547 nm. Arbitrary values obtained were compared with a series of standard solutions (1,1,3,3-tetramethoxypropane). Results were expressed as μmol/g of wet weight of esophagus.

Hydroxyproline Determination

Esophageal tissue samples for hydroxyproline determination were washed with 0.9% saline and dried for 72 h at 100°C. Hydroxyproline levels were determined spectrophotometrically using Woessner's method [26] after weighing and hydrolyzing the samples in concentrated hydrochloride acid (12M HCl) at 130°C for 3 h. Each sample was adjusted to 1 mL volume and centrifuged at 3000 *g* for 15 min before extracting the supernatant. After adding isopropanol to an equal volume of supernatant, further centrifugation was performed at 2500 *g* for 10min. Serial dilutions of pure L-hydroxyproline (4-hydroxy-l-proline, Sigma Chemical Co., St. Louis, MO) were used as a standard. Standards and samples were read at 557 nm on a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan). Hydroxyproline concentrations of the samples were calculated using the absorbance-concentration curve of standard hydroxyproline solutions. Results were expressed as mg/g of dry tissue weight. All samples were assayed in duplicate.

Statistical Analyses

All values are expressed as mean ± SD. Statistical comparisons of more than two groups were performed with a one-way analysis of variance (ANOVA). Differences between groups were analyzed using the post-ANOVA (Student-Newman-Keuls) test. The Mann-Whitney U test was used to detect significant differences between histologic grading scales. Statistical significance was accepted at $P < 0.05$.

RESULTS

Five rats (one in group 2, one in group 3, and three in group 5) died in the early postoperative period. An autopsy revealed a perforation at the site of the isolated esophageal segment in all animals. These animals

TABLE 1

The Histopathologic Collagen Scoring System. A Collagen Score is the Sum of the Scores in Each of the Three Categories Shown

Histopathologic evaluation criteria	Score
Submucosal collagen deposition	
None	0
Mild (submucosal collagen < 2× muscularis mucosa thickness)	1
Marked (submucosal collagen > 2× muscularis mucosa thickness)	2
Muscularis mucosa injury	
None	0
Yes	1
Muscular layer injury and collagen deposition	
None	0
Mild (collagen deposition around the smooth muscles)	1
Marked (collagen deposition around smooth muscles and replacement of muscles with collagen)	2

were excluded from further analysis and the results are therefore based on the remaining 55 rats.

Histopathology

Histopathologic examination of the esophageal wall did not reveal any lesion in group 1 rats (Fig. 1A). Stenosis of the esophageal wall with an increase in wall thickness and decrease in lumen diameter was observed in all animals in group 2 (Fig. 1B). The stenosis index (SI) results are shown in Fig. 2. SI was significantly higher in group 2 compared with all other groups. SI was significantly lower in both dexpanthenol and Y-27632 treated groups compared with group 2. There was no statistically significant difference in SI between groups 3 and 4, and between groups 5 and 6. Masson's trichrome staining showed an intensified collagen deposition in the submucosa and muscular layer of the esophageal wall in group 2 (Fig. 3). Mean collagen deposition scores (CS) are shown in Fig. 4. In dexpanthenol and Y-27632 treated groups, the scores were significantly lower than in the control group. Mean CS were significantly lower in group 4 compared with group 3 but there were no significant differences between groups 5 and 6.

Biochemical Results

MDA results are shown in Fig. 5. MDA concentrations were significantly higher in group 2 than in group 1. In groups 3, 4, 5, and 6, MDA concentrations were markedly lower than in group 2. There were no significant differences between groups 3 and 4, and between groups 5 and 6. Hydroxyproline levels were significantly higher in group 2 compared with group 1 (Fig. 6). Hydroxyproline levels were significantly lower in both dexpanthenol and Y-27632 treated groups compared with group 2. There were no statistically significant differences between groups 3 and 4, and between groups 5 and 6.

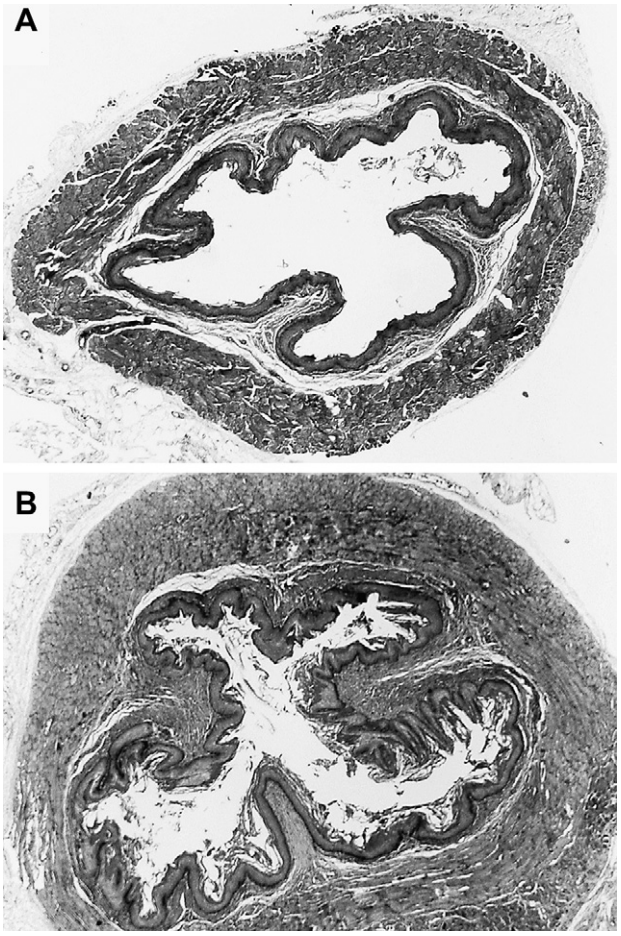

FIG. 1. (A) Microscopic appearances of the distal esophageal segment in group 1. Note the relatively normal histology of the rat esophagus. (B) Thickened wall and narrow lumen in a rat with caustic esophageal injury (group 2) (H and E, ×40).

DISCUSSION

In this experimental study, dexpanthenol and Y-27632 both demonstrated beneficial effects on stricture formation after caustic esophageal injury. Both agents significantly decreased the severity of histologic lesions, as well as producing a marked reduction in tissue concentrations of MDA and hydroxyproline.

Ingestion of acid or alkaline corrosive substances remains a major health hazard in children in developed and developing countries [2]. Mortality is high and lifelong consequences are often devastating [1, 3, 4]. During the early stages of injury after a caustic esophageal burn, there is a decrease in tissue perfusion and an increase in the breakdown of cellular membranes by lipid peroxidation. Reactive oxygen

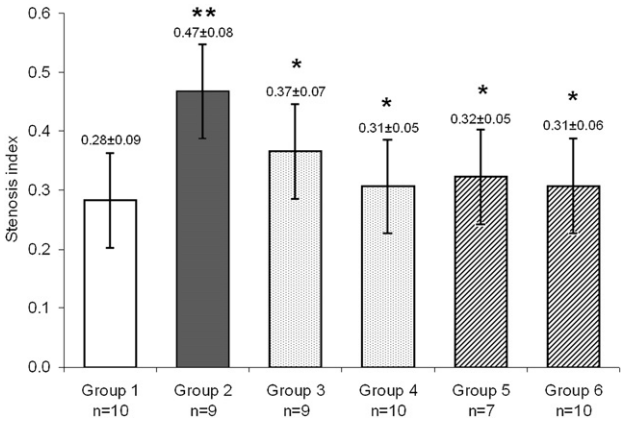

FIG. 2. Esophageal stenosis index results. $^{**}P < 0.05$ compared with group 1 (sham group), $^{*}P < 0.05$ compared with group 2 (control group).

radicals may play an important role in this early inflammatory phase of esophageal damage [27, 28]. The development of dense scar tissue at the injury site is a later consequence of caustic esophageal injury [10]. Once a stricture has developed, clinical management is even more demanding, requiring procedures such as repeated dilatations, esophageal stenting, and, sometimes, replacement of the esophagus with an intestinal conduit. There is no universally accepted medical treatment to avoid esophageal stricture formation [1, 3, 4]. Research has focused on the healing of the burned esophagus and reduction in collagen formation [9, 10]. In animal experiments, a variety of different medications, such as erythropoietin, caffeic acid phenethyl ester, mitomycin C, ketotifen, and resveratrol have been used in attempt to prevent esophageal stricture formation after caustic ingestion [6–10]. We investigated the role of dexpanthenol and Y-27632, two different agents with different modes of action. An esophageal stricture typically develops 3 to 4 wk after ingestion of the corrosive substance [4]. Thus, the rats in our experiment were sacrificed 22 d after caustic injury to investigate the outcome of treatment regimens on stricture formation. In our study, ingestion of 50% sodium hydroxide resulted in significantly greater collagen deposition and significantly higher SI scores compared with sham operated controls. These histologic changes were accompanied by significant increases in esophageal tissue MDA and hydroxyproline concentrations, indicating increased lipid peroxidation and total tissue collagen deposition, respectively.

Treatment with dexpanthenol led to a significant reduction in both histologic and biochemical indices of injury but these parameters (SI, MDA, and hydroxyproline concentrations) were not significantly different in comparison with sham operated controls, which is particularly encouraging. Two different dexpanthenol doses were studied and although SI, CS, MDA, and hydroxyproline levels were lower in the higher dose group, only the difference in CS was statistically significant.

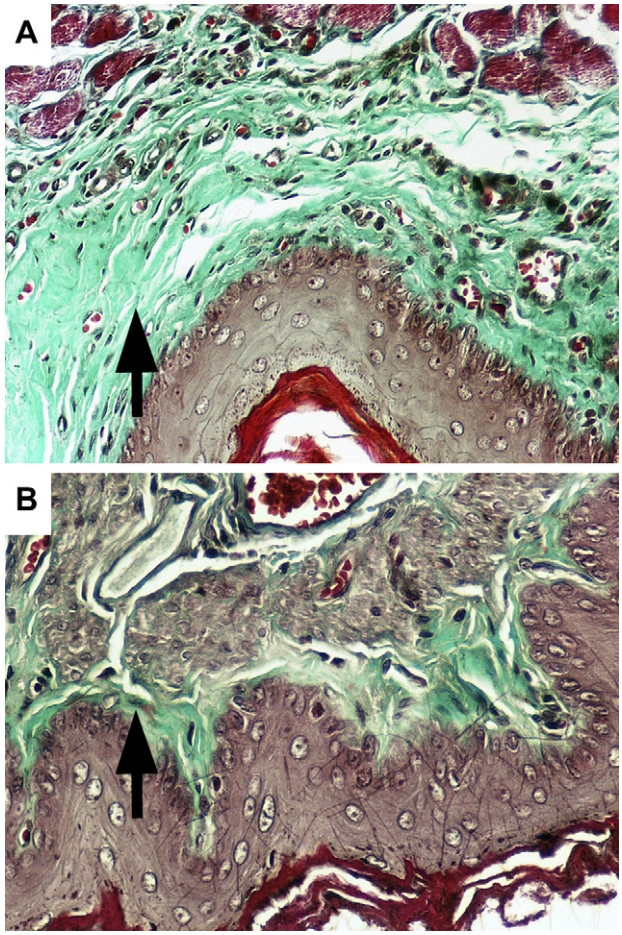

FIG. 3. (A) Excessive collagen deposition in the submucosal and muscular layers of the esophagus (arrow) in a stenotic esophagus. (B) Collagen deposition (arrow) was reduced in both dexpanthenol and Y-27632 treated groups. (Masson's trichrome, ×400). (Color version of figure is available online.)

Dexpanthenol is used topically as an adjunct in the treatment of various skin and mucosal lesions such as in scars, burns, leg ulcers, and anal fissures. Dexpanthenol can be given systemically, for example in the treatment of postoperative ileus and burning feet syndrome [11]. Both oral and parenteral dexpanthenol are oxidized enzymatically to pantothenic acid, which is widely distributed in tissues, mainly as coenzyme A [29, 30]. Pantothenic acid protects against cell damage produced by oxygen free radicals. It also increases the biosynthesis of glutathione, ATP, and coenzyme A, all of which help to decrease tissue damage [31, 32]. This may be one mechanism by which dexpanthenol reduced esophageal stricture formation in this experimental animal model.

The effects of Y-27632 in this experimental model of caustic esophageal stricturing were also impressive. SI, CS, MDA, and hydroxyproline concentrations were

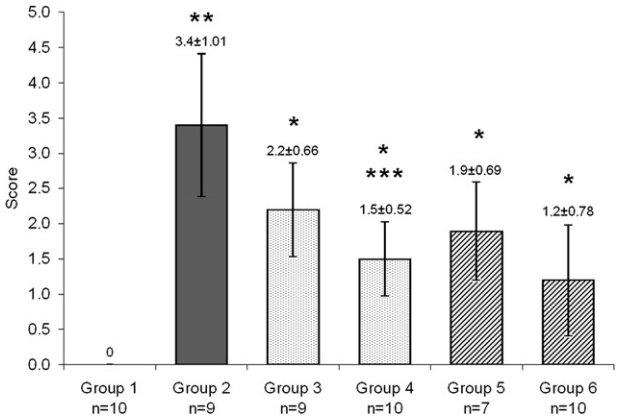

FIG. 4. Collagen deposition scores. $^{**}P < 0.05$ compared with group 1 (sham operated), $^{*}P < 0.05$ when compared with group 2 (controls). $^{***}P < 0.05$ compared with group 3 (50 mg/kg/d dexpanthenol).

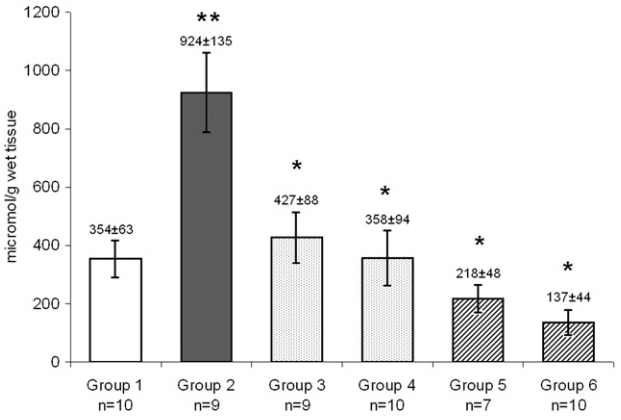

FIG. 5. MDA concentrations. $^{**}P < 0.05$ compared with group 1 (sham operated), $^{*}P < 0.05$ compared with group 2 (controls).

significantly lower after treatment with Y-27632 compared with controls. Further, SI, CS, and hydroxyproline concentrations were not significantly different compared with sham operated animals. Although all these histologic and biochemical parameters were lower in the higher dose treatment group, these differences were not statistically significant. Y-27632 treatment therefore showed beneficial effects on the healing after caustic esophageal injury.

The mechanism of Rho/Rho-kinase pathway activation in burns remains to be determined. One possibility is that complex factors, including proinflammatory mediators and cytokines, release activated Rho protein *via* G protein coupling receptors on cell membranes, followed by activation of Rho-kinase. In this context, it should be noted that Y-27632 has been shown to depress the striking elevation in serum levels of proinflammatory cytokines (interleukin-6, keratinocyte chemoattractant, and granulocyte colony-stimulating factor) in ischemia/reperfusion injury [33]. There is also evidence that Rho-kinase is activated in response to wounding, and that inhibition of Rho-kinase by Y-27632 enhances wound closure by modulating cell migration, proliferation, cell-matrix adhesion, and cell-cell adhesion in human corneal epithelial cell cultures [34]. Activated fibroblasts, or myofibroblasts, are crucial players in tissue remodeling and wound healing. If fibroblast activity is pathologically prolonged, hypertrophic scar formation and wound contractures may arise [35]. Inhibition of Rho-kinase has been shown to prevent myofibroblastic differentiation [36]. In addition, inhibition of Rho-kinase with Y-27632 has been reported to suppress actin stress fibers and focal adhesion formation, thereby stimulating wound closure of bronchial epithelial cells [21]. Rao et al. showed that Y-27632 decreased cell migration in intestinal epithelial cells [37]. Exposure of Y-27632 also reduces burn-induced hyperpermeability and albumin leakage [38] up-regulates eNOS expression and phosphorylation in rats [39]. These effects may collectively contribute to the protective effect of Rho-kinase inhibition on esophageal wound healing after caustic injury.

Reactive oxygen radicals may increase tissue damage after caustic esophageal injury [27]. This type of injury is associated with increased lipid peroxidation and a decrease in antioxidant enzyme levels [40]. Free radicals also indirectly accelerate the local infiltration and activation of polymorphonuclear leukocytes, which initiate an inflammatory process that exacerbates esophageal mucosal injury after caustic ingestion [4]. Y-27632 has been shown to inhibit superoxide production by activated human neutrophils [41] and to suppress neutrophil accumulation [33]. Y-27632 may also reduce the production of reactive oxygen species by suppressing NAD(P)H oxidase expression [39]. Together, these effects may lead to the inhibition of local inflammatory responses and diminished esophageal burn injury.

In conclusion, our results suggest that Rho-kinase plays an important role in the healing of caustic

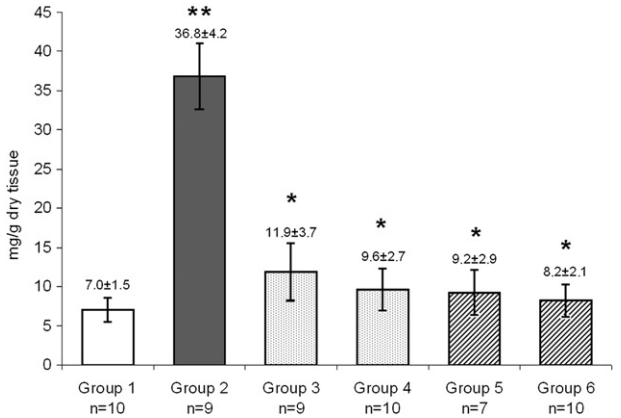

FIG. 6. Hydroxyproline results. $^{**}P < 0.05$ compared with group 1 (sham operated), $^{*}P < 0.05$ compared with group 2 (controls).

esophagitis, and that the Rho-kinase inhibitor, Y-27632, might be a potential therapeutic agent in the management of caustic injuries. Our findings also suggest that dexpanthenol may be able to prevent stricture formation after caustic (alkaline) esophageal injury. Its track record on safety, the availability of both enteral and parenteral forms, and the cost of dexpanthenol make this a particularly attractive candidate for further evaluation in this context.

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