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A novel approach for preventing esophageal stricture formation: olmesartan prevented apoptosis

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Abstract: Accidentally ingested corrosive substances can cause functional and structural damage to the esophageal tissue resulting in stricture formation. It has been reported that the administration of olmesartan (OLM) can have anti-inflammatory, antifibrotic and antiapoptotic effects on injured tissue. The aim of our study was to check if OLM could prevent formation of scars in the corrosive esophageal burn model. Fifty-one Wistar Albino rats were divided into six groups: Control, Sham, OLM, Sham + OLM, Burn, and Burn + OLM. Olmesartan (5 mg/kg) was given by gavage once per day for 21 consecutive days after injury. The morphology of the esophagus was assessed after Masson trichrome staining, and apoptosis was evaluated using the terminal deoxynucleotidyl transferased UTP nick end labeling (TUNEL) method. The serum nucleosomes (as an indicator of apoptosis), serum p53 protein, and esophageal tissue p53 protein levels of each group were measured by immunoassays. Muscularis mucosa damage, submucosal collagen deposition, and tunica muscularis injury in the Burn + OLM group decreased significantly compared with the Burn group ($p < 0.05$). Similarly, the number of apoptotic cells in the Burn + OLM group decreased compared with the Burn group ($p < 0.05$). Serum levels of nucleosomes and p53 and tissue of p53 protein did not differ between the groups. Exogenously administered OLM can effectively prevent the occurrence of esophageal strictures caused by corrosive esophageal burns. *(Folia Histochemica et Cytobiologica 2014, Vol. 52, No. 1, 29–35)*

Key words: corrosive esophageal burn; stricture; olmesartan; protection; apoptosis; TUNEL; nucleosomes; p53

Introduction

Caustic esophageal burns in both children and adults remain an important cause of morbidity and mortality around the world [1]. The burns can occur in the mouth and larynx and can cause extreme damage to the organs, resulting in loss of voice, difficulty in swallowing, and airway obstruction [1]. Long-term complications include the deposition of collagen and stricture formation. The latter can also increase gastroesophageal reflux and predispose to esophageal carcinoma [2]. Thus, one of the most important goals in treating of corrosive esophageal burns is to prevent the stricture formation. Several types of nonsurgical stricture techniques have been proposed, including dilatation, esophageal stent placement, total parenteral nutrition, steroids, and antibiotic therapy [1, 2].

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However, none of these treatment modalities seems to be effective in the treatment of severe caustic esophageal burns [1, 2].

Angiotensin II and its receptor type 1 (AT1R) together with its downstream mediators are involved in immune and inflammatory responses and they can contribute to the generation of oxidative stress, regulation of cell growth and cell death and promotion of tissue remodeling. Inhibition of AT1R signaling can attenuate cellular stress and provide beneficial effects to the injured tissue [3, 4]. Olmesartan (OLM) is AT1R antagonist. In diabetic rat model of liver fibrosis OLM was found to be the most protective among tested AT1R blockers [5]. However, the effects of OLM on esophageal tissue regeneration and fibrosis after corrosive esophageal burns have yet not been studied.

Therefore, the aim of our study was to evaluate the effects of OLM on wound healing and stricture formation in the rat esophagus after caustic injury.

Material and methods

Animals and experimental protocol. The Trakya University guidelines for the care and use of laboratory animals were followed, and ethical committee consent was obtained for the study. Fifty-one female Wistar Albino rats weighing 200–250 g each were randomly assigned to six groups: Control (n = 6), Sham (n = 8), OLM (n = 8), Sham + OLM (n = 8), Burn (n = 12), and Burn + OLM (n = 9). The rats in Sham and Sham + OLM groups underwent esophageal dissection only, while the rats in Burn and Burn + OLM groups underwent both esophageal dissection and experimental esophageal burn formation.

The rats were anesthetized with ketamine and xylazine administered *i.p.* (50 mg/kg and 10 mg/kg, respectively). Caustic esophageal injury was produced using the Liu and Richardson [6] modification of the experimental model of Gehanno and Guedon [7]. Briefly, after a median laparotomy, a 1.5-cm segment of the distal esophagus was exposed, and an 8 Fr feeding catheter with an outer diameter of 0.22 cm was placed into the distal esophagus via the oral route. A similar catheter was placed into the distal esophagus via gastrotomy. The catheters were secured, and an isolated segment of the distal esophagus was obtained. The distal catheter was clamped, and the isolated segment was distended by infusion of 25% NaOH at an intraluminal pressure of 10 cm H_2O for two minutes, until a slight translucency of the esophageal wall and branching of the vessels were noted. Following this procedure, the distal catheter was unclamped, and the esophagus was rinsed with 40 mL of distilled water. The gastrotomy and laparotomy were subsequently closed.

Immediately after the formation of the esophageal burn in the Burn + OLM group, the administration of OLM (5 mg/kg daily) through gavage began and was continued for 21 days. A similar OLM treatment was applied to the OLM and Sham + OLM groups. After the surgery, all animals were kept on a standard rodent pellet diet with tap water *ad libitum*, and 10 mL of 0.9% NaCl was administered subcutaneously for the first three postoperative days. Twenty one days after the initial treatment, rats were anesthetized and blood was collected for biochemical assays. The esophagus was opened longitudinally, and the injured area was removed and divided into two pieces. One sample was fixed in 4% neutral-buffered formaldehyde solution, whereas the other sample was kept at –80°C until further biochemical analyses. All the rats were subsequently euthanized with a high dose of phenobarbital.

Assessment of esophageal tissue. Esophageal tissues were examined under a light microscope at the Trakya University Faculty of Medicine, Histology and Embryology Department's Light Microscopic Laboratory. For this purpose, the esophageal tissues were fixed for four days in neutral-buffered 10% formalin solution. Then, tissues were washed for two days in 70% alcohol and processed into paraffin blocks using a standard procedure. Using a Leica RM-2245 microtome, 6 μm thick sections were stained using the Masson trichrome method. The deposition of submucosal collagen in the esophageal wall and muscular mucosa and the depth of the injury were assessed and scored semi-quantitatively as described previously [8].

Evaluation of apoptotic cells by the TUNEL method. The apoptosis of the fibroblasts of esophageal submucosa was evaluated using the terminal deoxynucleotidyl transferased UTP nick end labeling (TUNEL) technique [9]. The 6 μm thick sections were placed in the oven at 37°C overnight, and afterwards were thoroughly cleared of paraffin and rehydrated in decreasing concentrations of ethanol. Thereafter, sections were incubated at room temperature for 15 minutes in proteinase K solution (20 mg/mL, Cat. no. 21627, Chemicon International, Temecula, CA, USA). The sections were washed with distilled water, and then in the solution of methanol in 3% H2O2 for five minutes to block the endogenous peroxidase activity. Later, the sections were incubated with the terminal deoxynucleotidyl transferase enzyme (Cat. no. S7100, Chemicon) in the oven at 37°C for one hour. The anti-digoxigenin sections were also washed with PBS three times for 10 minutes. Diaminobenzidine dissolved in PBS was applied at room temperature for 30 minutes. The sections were washed again in distilled water for ten minutes, and methyl green staining was performed. The sections were then dehydrated with toluol for 2–3 minutes, cover-slipped, and evaluated by light microscopy. To count the TUNEL-positive cells in the esophageal tissue, the ocular micrometer compatible with an Olympus BX51 microscope was used. TUNEL-positive cells were counted

under a 100-square ocular micrometer (eye piece graticule; eyepiece ×10, objective ×40). In each section, the number of cells in ten different, randomly selected areas were counted, and the arithmetic mean was calculated. For the ×40 magnification, the 100-square micrometer area was determined by means of a micrometer slide. Finally, all counts were expressed as the number of TUNEL-positive cells per unit area (mm^2).

Determination of nucleosome concentration in the serum. The serum nucleosomes level (mono- and oligonucleosomes as indicators of apoptosis) was determined using the ELISA kit (Cat. no. 11774425001; Roche, Maharashtra, India), according to the manufacturer's instructions.

Determination of p53 protein contents in esophageal tissue and the serum. Prior to biochemical measurements, the esophageal tissue samples were weighted and added to 10-fold quantity of cold potassium phosphate buffer (pH 7.4) and homogenized using a glass homogenizer at 4°C. The homogenates were centrifuged at 10.000 rpm at 4°C, and the supernatants were separated. The concentrations of p53 protein in esophageal tissue homogenates and in the serum were assessed using the ELISA kit (Cat. no. 11828789001; Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

Statistical analysis. The data was analyzed using a commercially available statistical software package (SPSS 15.0, SPSS Inc., Chicago, IL, USA). The normal distribution of the variables was tested using a one-sample Kolmogorov-Smirnov test. For variables with normal distribution, the differences among the groups were compared using one-way ANOVA followed by the Bonferroni post-hoc test if a significant difference was found. The Kruskal-Wallis test was used for non-normal distributed data. Dual comparisons between the groups that were significantly different were evaluated using the Mann-Whitney U test. The results are presented as means ± SEM, and p values less than 0.05 were regarded as statistically significant.

Results

Assessment of esophageal tissue stained by Masson trichrome method

Changes that occurred in the esophageal tissue after the corrosive esophageal burns were examined according to the criteria and scores shown in Table 1. In the Burn group the muscular layer exhibited a higher number of fibroblasts, increased collagen deposition between muscle bundles and reduced thickness as compared with the intact esophagus of control group (Figure 1B and 1A, respectively). However, in the Burn + OLM group collagen fiber deposition and number of fibroblast between muscle cells were reduced as compared with the Burn group (Figure 1C and 1B, respectively). The results of semiquantitative histopathologic evaluation of esophageal tissues showed that the submucosal collagen deposition, muscularis mucosa damage, and tunica muscularis damage scores of the Burn + OLM group were found to be significantly lower than those of the Burn group ($p < 0.005$, $p < 0.005$, and $p < 0.002$, respectively; Table 2).

Table 1. Histopathological evaluation criteria and scores of esophageal damage according to Türkyilmaz et al. [11]

Criteria	Score
Increased submucosal collagen deposition	
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 +
Presence of injury in the muscularis mucosa	
None	0
Present	1 +
Presence of injury and collagen deposition in the tunica muscularis	
None	0
Mild (collagen deposition around myocytes)	1 +
Marked (same as mild, with collagen deposition replacing some myocytes)	2 +

Assessment of apoptosis in the esophageal tissue

The results of the TUNEL staining of fibroblasts in the submucosa of esophageal wall are shown in Table 3 and Figure 2. The average number of apoptotic cells was significantly reduced in the Burn + OLM group as compared to the Burn group ($p < 0.005$; Table 2). At the same time, the average number of apoptotic cells in the Burn and Burn + OLM groups were higher than in the other groups (Control, Sham, OLM, and Sham + OLM; $p < 0.002$ and $p < 0.003$, respectively; Table 2).

Biochemical measurements

The serum concentrations of nucleosomes and p53 protein as well as the contents of p53 protein in esophageal tissue were similar in all experimental groups and did not differ significantly (Table 4).

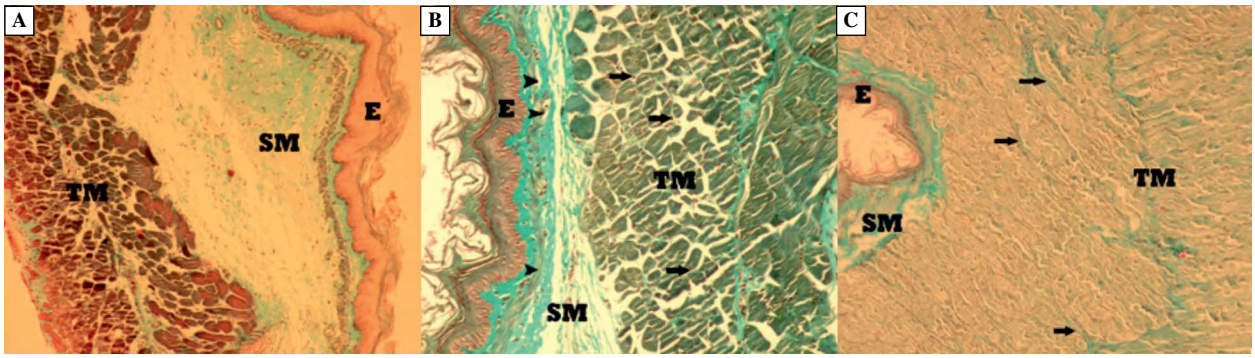

Figure 1. Histologic appearance of esophagus stained by Masson trichrome method. **A.** Normal esophagus (control group), ×200; **B.** Burn group: in the muscle layer an increased number of fibroblasts is seen (*arrows*) and the thickness of the muscularis mucosa (*arrowheads*) is reduced, ×400; **C.** Burn + OLM group: the reduction of connective tissue (*arrows*) in the muscle layer of esophageal wall is clearly visible, as well as reduced number of fibroblasts, ×400. Symbols: E — epithelium; SM — submucosa; TM — tunica muscularis

Table 2. Semiquantitative histopathologic evaluation of esophageal tisues based on Masson trichrome staining

	Control	Sham	OLM	S+OLM	Burn	Burn+OLM
Submucosal collegen deposition	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.58±0.19*	0.78±022
Muscularis mucosa injury	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.00±0.00*	0.44±0.18
Tunica muscularis injury and collagen deposition	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.67±0.14**	0.56±0.24
Total score					4.25 ± 0.33**	1.89± 0.51

Description of groups as in Methods. Values are expressed as mean ± SD, scores were obtained according to the criteria presented in Table 1. *, **significantly different from Burn+OLM group, $p<0.005$ and $p<0.002$, respectively.

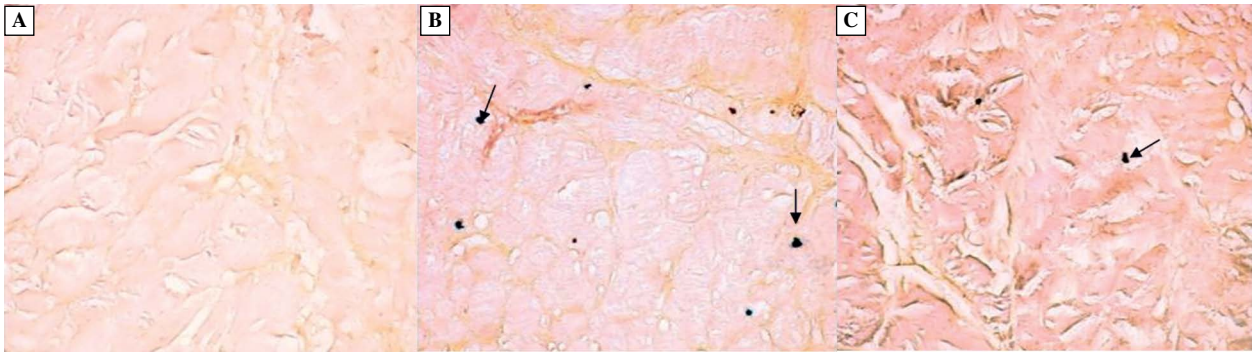

Figure 2. Presence of apoptotic cells in esophageal tissue visualized by TUNEL method. **A.** Control rat, lack of apoptotic cells; **B.** Burn group: increased number of TUNEL-positive cells; **C.** Burn + OLM group: lower number of TUNEL positive cells as compared with the Burn group. Symbols: *arrows*, TUNEL positive cells. ×400

Table 3. The number of apoptotic cells in esophageal tissues of the studied groups

Control	Sham	OLM	S+OLM	Burn	Burn+OLM
3.32±0.75	3.64±0.90	3.82±0.70	3.50±1.38	56.17±0.90*#	31.22±1.17†

Apoptosis was assessed by the TUNEL method as described in Methods. Values are expressed as mean ± SD. Description of groups as for Table 2. *significantly different from Control, Sham, OLM and S+OLM groups, $p < 0.002$; # $p < 0.005$ compared with Burn+OLM; † significantly different from Control, Sham, OLM and S+OLM groups, $p < 0.003$

Table 4. The concentrations of nucleosomes and p53 and tissue level of p53 protein in esophageal tissue

	Sham	OLM	S+OLM	Burn	Burn+OLM
Serum nucleosomes (mU/ml)	7.17±3.64	3.43±0.59	2.45±0.32	4.68±0.96	24.93±15.57
p53 tissue content (pg/g wet tissue)	3008.3±481.5	3829.8±1104.8	2446.0±438.5	2821.9±594.5	2028.6±571.4
p53 serum level (pg/ml)	47.84±10.46	96.01±32.87	42.18±14.21	156.54±61.91	40.88±15.70

Description of groups as for Table 2. Serum concentration of nucleosomes and p53 protein as well as p53 protein content in tissues were measured as described in Methods. Values are expressed as mean ± SD. There were no statistically significant differences between groups, $p > 0.05$

Discussion

Esophageal burn caused by corrosive substances initially leads to acute necrosis and thrombosis with consequent ischemia and hypoxia, which are followed by the inflammation and oxidative stress [1, 2]. Prolonged stressful conditions are caused by the release of proinflammatory cytokines (and growth factors) as well as the exposure to reactive oxygen species (ROS). Both can result in an excessive activation and proliferation of fibroblasts but also in increased rate of cell death [3, 10]. By the second and third week after the caustic injury, collagen deposition begins and fibrosis followed by stricture formation may occur [2]. It is well documented that after the cell injury, the renin-angiotensin system, particularly the signaling mediated by angiotensin II receptor type I, is essential for the activation of NADPH oxidase, stimulates secretion of pro-inflammatory and profibrotic cytokines and exerts a direct mitogenic effect on fibroblasts [3, 4]. Thus, we decided to investigate the putative influence of treatment with olmesartan, an AT1R blocker, on fibrosis and apoptosis in the rat model of esophageal caustic burn. The administration of OLM started immediately after the injury and was continued during the next 21 days, including second and third week of tissue repair, *i.e.* recovery period during which collagen deposition and formation of strictures occur.

The results of the present study provide the first demonstration that the treatment with OLM can effectively prevent fibrosis and limit the damage to the esophageal tissue in the animal model of caustic burn. We also demonstrated that administration of OLM can reduce proliferation of fibroblasts and deposition of collagen induced by the injury. Moreover, we found that OLM can have anti-apoptotic properties and significantly limit injury-evoked apoptosis in the esophagus.

In recent years several experimental studies have shown that OLM-mediated AT1R inhibition can provide beneficial effects in respect to the excessive collagen deposition which leads to fibrosis and tissue remodeling. OLM was found to inhibit peritoneal fibrosis and loss of mesothelium in a laboratory model of hypertensive rats subjected to continuous peritoneal dialysis [11]. Inhibition of AT1R in a rat model of nonalcoholic steatohepatitis resulted in a significant reduction of the activation of stellate (Ito) cells, oxidative stress and the expression of liver fibrosis-related genes: transforming growth factor-β1 (TGF-β1) and collagen genes [12]. OLM was also successfully tested as a potential antifibrotic drug in another experimental model of liver fibrosis [13]. It was shown that the inhibition of AT1R suppressed the proliferation of stellate cells, collagen synthesis and expression of profibrogenic cytokines such as TGF-β1 and connective tissue growth factor (CTGF) by activated stellate cells. AT1R antagonism appeared to attenuate collagen synthesis, fibrosis and structural remodeling in rabbit model of atrial fibrillation and it was proposed that OLM acted through the inhibition of p38MAPK/ERK pathway in cardiac fibroblasts [14]. Furthermore, in the canine model of atrial fibrillation, interstitial fibrosis was caused by the AT1R-dependent up-regulation of strong mediator of ECM synthesis, CTGF, and this effect was partially abolished by OLM [15].

The anti-fibrotic properties of OLM cannot be dissociated from its anti-apoptotic and anti-necrotic effects which play a protective role in injured tissues. For instance, OLM administered after a myocardial infarction attenuated injury-evoked up-regulation of BAX protein, Fas receptor and Fas ligand [16]. Similarly, the induction of TNF-α expression in the rat model of steatohepatitis was limited by the inhibition of AT1R signaling by OLM [15]. In a study of experimental autoimmune myocarditis OLM prevented apoptosis by decreasing the levels of mediators of oxidative stress, endoplasmic reticulum stress, and cardiac inflammatory mediators [17]. It was also documented that anti-apoptotic action of OLM in perivascular injury and hepatic steatosis can be mediated by inhibition of the apoptosis signal regulating kinase 1 (ASK1) [18].

The presented study is the first one which demonstrated anti-apoptotic effect of OLM on injured

esophageal tissue. The number of TUNEL-positive apoptotic cells decreased in the esophagus of OLM -treated animals, however, the levels of the p53 protein were similar between the experimental groups. The local tissue injury did not affect serum levels of p53 protein and nucleosomes in the OLM-treated animals. It was documented that the activation of p53 protein (and its downstream proteins) in cultured myocytes correlated with the activation of renin-angiotensin system mediated by AT1R [19]. The ability of p53 protein to bind DNA and induce apoptosis in these cells was abolished by the treatment with AT1R blocker, losartan [19]. Thus, the pro-apoptotic action of p53 is mediated rather by changes in its activity and binding properties than quantity. Moreover, the p53 protein has a very short half-life period (several minutes) [20]. These two facts can explain why we did not find any differences between experimental groups in the tissue and serum levels of the p53 protein.

Tissue-protective effects of OLM were demonstrated in studies carried out on rats subjected to subtotal nephrectomy. Morphological and functional symptoms of progressive renal injury induced in these rats were alleviated by the inhibition of AT1R [21, 22, 23]. Similarly, the treatment with OLM significantly attenuated the number of TUNEL-positive cells in the animal model of progressive glomerular injury [23] and in the kidneys of hypertensive rats [24]. Moreover, in both cited studies, OLM-reduced apoptosis was associated with lower degree of interstitial fibrosis or glomerular sclerosis, respectively. Authors of the above-mentioned studies suggested that beneficial role of OLM was associated rather with its antioxidant properties than antihypertensive action on renal cardiovascular system. The latter suggests that the anti-fibrotic and anti-apoptotic effects of OLM administration in esophageal caustic burn observed in our study could be also attributed to the suppression of oxidative stress by AT1R antagonism. This hypothesis is in agreement with the results of previous studies which demonstrated partially successful treatment of caustic esophageal burns by increasing the antioxidant capacity of the tissue [25–35]. The present study provides new evidence that the renin-angiotensin system can be a promising target for the treatment of esophageal burns caused by corrosive agents.

In conclusion, our results confirm that administration of OLM can reduce pro-fibrotic and pro-apoptotic stimuli evoked by caustic esophageal burn. Based on the results of ours and other studies we demonstrate that the treatment with OLM can have direct impact on the formation of stenosis in esophageal caustic burn, possibly due to anti-inflammatory and antioxidant properties of OLM. Further studies are needed to unravel the mechanisms of anti-fibrotic and anti-apoptotic action of OLM in the esophageal tissue.

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