Morphofunctional Analysis of Experimental Model of Esophageal Achalasia in Rats

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We carried out a detailed analysis of rat model of esophageal achalasia previously developed by us. Manifest morphological and functional disorders were observed in experimental achalasia: hyperplasia of the squamous epithelium, reduced number of nerve fibers, excessive growth of fibrous connective tissue in the esophageal wall, high contractile activity of the lower esophageal sphincter, and reduced motility of the longitudinal muscle layer. Changes in rat esophagus observed in experimental achalasia largely correlate with those in esophageal achalasia in humans. Hence, our experimental model can be used for the development of new methods of disease treatment.

Key Words: *lower esophageal sphincter; esophageal achalasia; experimental model; S-100 protein; neurodegeneration*

Esophageal diseases rank among the most incident diseases of the gastrointestinal tract. Esophageal achalasia (EA) is a progressive neurodegenerative disease consisting in impairment of relaxation of the lower esophageal sphincter (LES) and reduction of propulsive motility of the esophagus [7]. The pathogenesis of EA originates from degenerative changes in neurons of the Auerbach's intermuscular nerve plexus responsible for smooth muscle relaxation in LES during swallowing [12]. The etiology of EA remains unclear until present. The neurotropic viruses and genetic liability are hypothesized to be involved in the disease development [5,13].

Therapeutic methods used in EA (drug therapy, pneumatic balloon dilatation of LES, surgical cardiomyotomy, *etc*.) are often ineffective and associated with serious complications [4,10]. Hence, the development of new effective methods for the treatment of EA is an important problem. This implies the need in an adequate experimental model of EA on laboratory animals.

We previously created an experimental model of EA on rats, improved in comparison with previous studies, and proved it to be adequate to the clinical picture of human disease [9]. For example, rats with experimental EA lost body weight and developed high pressure in the LES and lower third of the esophagus [2]. However, more detailed morphofunctional analysis of this EA model is needed for validating its usefulness for the development of new therapeutic methods. This analysis became the aim of this study.

MATERIALS AND METHODS

Esophageal achalasia was simulated in albino rats (180-200 g). The study was carried out in 3 groups of animals: intact controls, sham-operated animals, and achalasia group. Animals of the latter group were operated using a previously developed method of

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modified operation for creation of experimental EA [2]. The operation consists in local pharmacological denervation of the abdominal portion of the esophagus by application of a neurotoxin (benzalconium chloride; 0.2% solution) with a protector preventing leakage of the neurotoxin to the abdominal cavity. Sham-operated animals were subjected to superior medial laparotomy and wound suturing. The animals were taken into experiment on day 41 after the operation.

Histological, morphometric, and immunohistochemical analyses of esophageal sections were carried out. The material was routinely fixed in 10% neutral formalin and embedded in paraffin. The sections (3-5 μ) were stained with hematoxylin and eosin and examined in a clear field under an Olympus BX51WI microscope at ×60. Morphometric analysis consisted in evaluation of the esophageal myofibril diameters using ImagePro software. A total of 20 circular and 20 longitudinal fibrils were analyzed per micropreparation. Immunohistochemical analysis was carried out by the indirect immunoperoxidase method using LSAB-kit (DAKO) with antibodies to S-100 protein (1:100, DAKO) and vimentin (1:80, DAKO). Since S-100 protein is expressed by Schwann cells, its immunohistochemical detection visualizes neurofilaments of the peripheral nervous system [1]. Vimentin is a component of collagen and procollagen and hence, a marker of fibrous connective tissue [11].

Esophageal contractility was studied by the standard myographic method on a Power Lab device fitted with MLT 050/D pickups (AD Instruments). A ring 2-4 mm wide was isolated from the LES and dissected in order to obtain a strip for registration of contractile activity of the circular muscle layer. A longitudinal strip 2-4 mm in diameter was dissected from the lower third of the esophagus for recording contractile activity of the longitudinal muscle layer. Esophageal preparations were placed into perfusion solution of the following composition (mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose. The solution was aerated with carbogen (95% O_2, 5% CO_2) throughout the entire experiment, pH was maintained at 7.3-7.4, and temperature at 20°C. Esophageal preparations were fixed vertically with one end to the tensometric pickup and the other to the immobile holder and plunged in separate vessels (20 ml) with perfusion solution and carbogen. Contractions of esophageal strips were induced by electrical stimuli (10 V, 50 msec, 1 min^{-1} frequency). The preparations were plunged in the vessels and optimal stretching of the muscle strips was attained over 40-60 min adaptation period.

The contractions were recorded and the results were processed using Chart 5.55 software (AD Instruments). The initial contraction force was expressed in grams, the duration of the shortening phase and half-relaxation phase in seconds. The data were statistically processed using Origin 7.5 software. The results are presented as $M \pm m$, the significance of differences between the values was evaluated by Student's t test ($p<0.05$).

RESULTS

Morphology of the esophagus in experimental EA. In humans, EA is characterized by manifest morphological changes observed mainly in the middle and lower thirds of the esophagus. These changes are as follows: reduced count or complete disappearance of the intermuscular plexus ganglia, inflammatory infiltration of the walls, accumulation of collagen fiber and fibrous connective tissue between the smooth muscle tissue bundles [8].

We carried out a morphological analysis of experimental EA in rats. Stratified squamous epithelium of the esophageal wall of intact and sham-operated rats had 3-6 layers with moderately pronounced keratinizaton. The basal layer of intact rat squamous epithelium consisted of one layer of flat cells, in sham-operated animals of 1-3 layers of cells (Fig. 1, *a*). The number of cell layers in rats with experimental EA increased up to 15, keratinization was more pronounced. The basal layer was thick and consisting of 6-7 layers of hyperchromatic cells (Fig. 1, *b*).

Morphometric analysis showed that the mean diameters of LES fibrils are similar in intact and sham-operated animals: circular 12.8±2.0 and 14.3±2.1 μ, respectively, and longitudinal fibrils 11.9±1.8 and 13.3±1.6 μ (Fig. 1, *c*). The mean diameters of esophageal fibrils in rats with achalasia were significantly (4-fold) greater: 48.6±7.12 and 50.3±7.98 μ, respectively (Fig. 1, *d*).

The next step of our study was immunohistochemical analysis of the esophagus with antibodies to S-100 protein and vimentin. Positive staining for S-100 protein in the submucous and intermuscular nerve plexuses was detected in intact and sham-operated rats. The reaction to vimentin was negative. A different picture was observed in rats with achalasia: no reaction to S-100 protein in the esophageal walls and bright staining of fibrous structures between the myofibrils and in the submucous layer with antibodies to vimentin.

Hence, experimental EA in rats is morphologically characterized by hyperplasia and hyperkeratosis of the multilamellar squamous epithelium, significant hypertrophy of the longitudinal and circular myofibrils in LES and lower third of the esophagus, reduced number of nerve fibrils in the intermuscular and submucous nerve plexuses, and fibrous connective tissue growth in the esophageal wall. Hence, the morphology

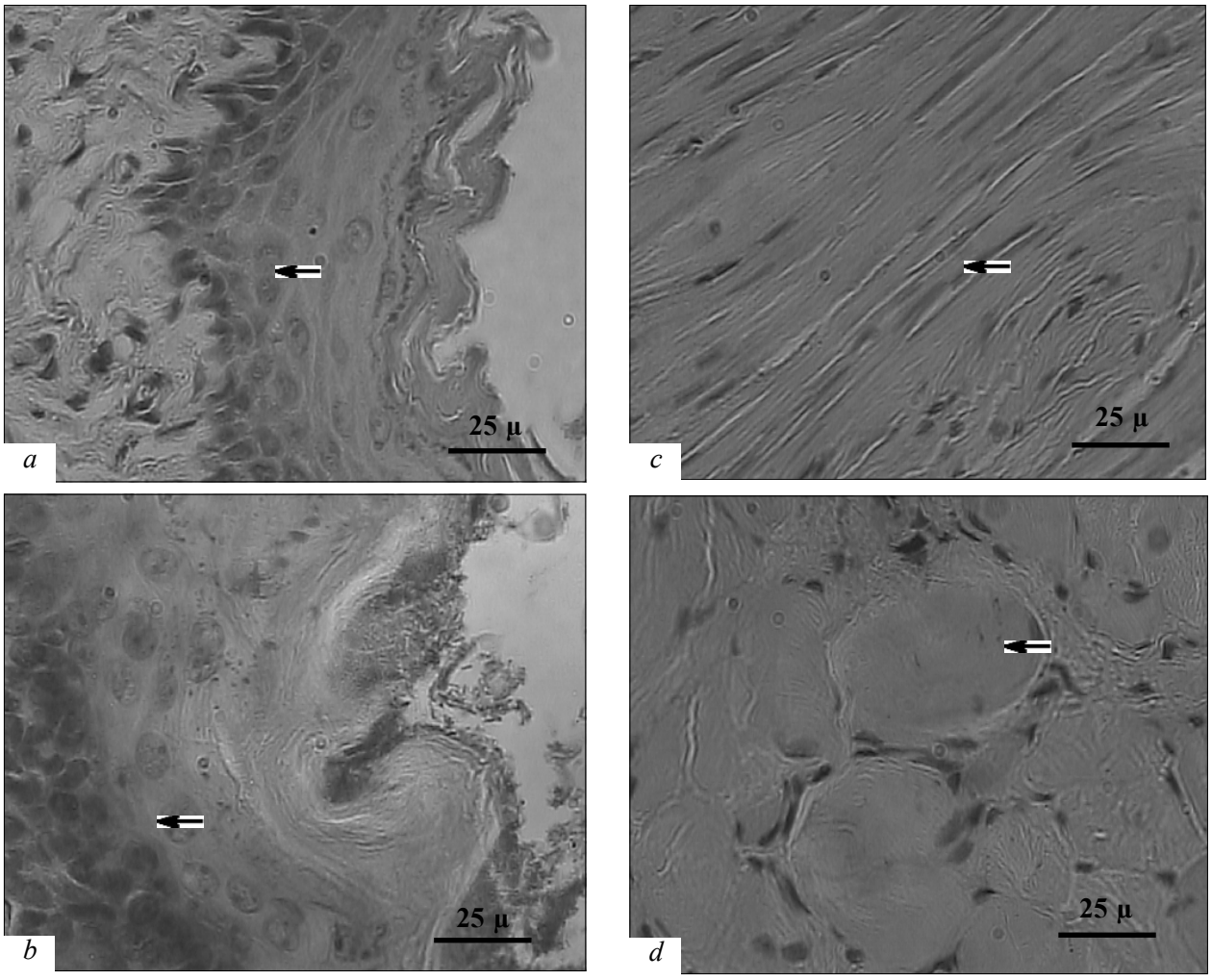

Fig. 1. The wall of the lower third of the esophagus in rat. Squamous epithelium in intact rats (*a*) and in rats with achalasia (*b*). Arrows show the basal layer of epithelial cells. Esophageal muscular membrane in intact rats (*c*) and rats with achalasia (*d*). Arrows show smooth muscle cells. Hematoxylin and eosin staining, ×60.

TABLE 1. Parameters of Evoked Contractile Responses of Esophagus Induced by Electrical Stimuli (*M*±*m*)

Parameters of contractile activity	LES		Lower third of esophagus			
	Normal values	EA	Normal values		EA	
	SCR	SCR	RCR	SCR	RCR	SCR
Amplitude, g	0.04±0.01	0.06±0.02	0.26±0.06	0.07±0.02	0.46±0.11*	0.06±0.02
Time of increase, sec	4.77±1.03	4.03±0.50	0.27±0.02	3.42±0.43	0.31±0.03	3.15±0.34
Time of relaxation (half-relaxation), sec	4.81±0.88	6.92±1.37	0.30±0.03	4.94±0.9	0.41±0.04	5.81±1.34
Number of experiments	12	9	13	12	12	10

Note. SCR: slow contractile response; RCR: rapid contractile response. *$p<0.05$ compared to normal values.

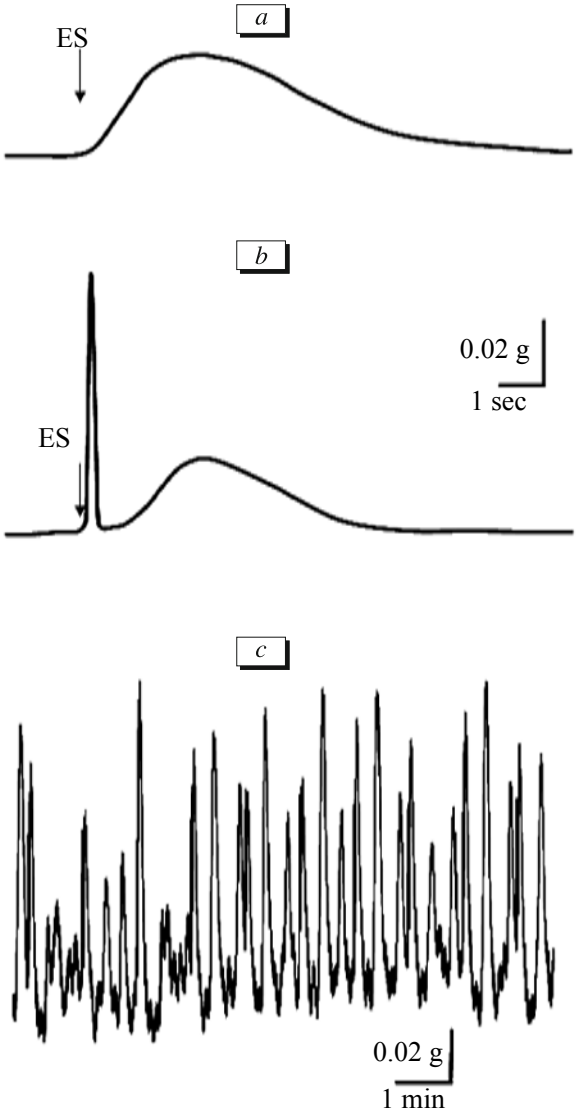

Fig. 2. Contractile activity of the esophagus. Mechanomyographic record of evoked contractile responses of LES (*a*) and lower third of the esophagus (*b*) induced by electrical stimuli (averaged for 20 realizations). Arrows show the moments of electrical stimulation (ES). An example of spontaneous contractile activity of the lower third of the esophagus (*c*): total duration of window: 9 min; frequency of activity: 3.2 min^{-1}. Results of a representative experiment.

of the esophagus in experimental EA in rats correlates with that in humans, which confirms that this model is adequate for studies of human EA.

Contractile activity of the esophagus in health and experimental EA. Two types of esophageal contractile responses to electrical stimulus were detected in intact rats: rapid and the subsequent slow responses (Fig. 2, *a*, *b*). The amplitude of the rapid response was significantly higher than that of the slow response for LES and the lower third of the esophagus ($p<0.05$). The parameters of induced contractile activity of the esophagus are presented in Table 1.

Slow contractile responses in the LES were observed in 92.3% cases, rapid ones in only 38.5% of experiments (n=13). Hence, slow responses are more typical of the LES, and therefore, only these responses were analyzed.

Spontaneous contractile responses, similar by shape to slow contractions induced by electrical stimulation, were observed in 7.7% cases. Biphasic contractile responses consisting of a rapid (present in 100% cases) and slow (present in 92.3% cases) phases were characteristic of the lower third of the esophagus (Fig. 2, *b*). Spontaneous contractile activity of the lower esophageal third was detected much more often than in LES: in 46.2% cases (Fig. 2, *c*). Biphasic pattern of contractile activity of the esophagus induced by electrical stimulation is presumably due to contractions of the smooth muscle cells under the effect of electrical stimulus (direct stimulation) and in response to the mediator release from the nerve terminals (indirect stimulation).

The parameters of contractile activity of esophageal strips from sham-operated animals virtually did not differ from those in intact rats ($p>0.05$). The contractile activity of the esophagus changed significantly in experimental EA.

Rapid contractile responses of LES in rats with achalasia were more frequent (46.2% observations) and slow ones rare (69.2%; n=13), while the amplitude of contractions did not differ much from that in intact animals ($p<0.05$; Table 1). Spontaneous slow contractile responses were observed in 15.4% cases, which was more often than in intact rats. The lower third of the esophagus was characterized (similarly as in intact animals) by biphasic contractile responses, but the incidence of rapid and slow responses was lower than normally (up to 92.3 and 76.9%, respectively). The rapid response amplitude was significantly higher than in intact rats ($p<0.05$), while the slow response amplitude was the same ($p>0.05$). Spontaneous activity of the lower third of the esophagus in EA was lower by amplitude and much more rare than in the control (in only 30.8% cases).

Hence, rapid evoked responses and spontaneous activity in LES were more incident in experimental EA than normally. This can be attributed to stimulation of smooth muscle cells because of degeneration of the esophageal inhibitory neurons. Reduced incidence of slow evoked responses can be due to neurotransmitter deficiency resulting from neuron degeneration [14] and elevated tone of LES in EA [2].

The rapid and slow evoked responses were more rare in experimental EA. Spontaneous contractility of the lower third of the esophagus was also lower than normally. This indicated impairment of the esophageal propulsive motility. These observations correspond to the clinical picture of EA [3]. A greater amplitude of

contractions of the lower third of the esophagus was presumably a compensatory reaction caused by the need to push a food lump through spasmodic LES.

Histological, immunohistochemical, and myographic studies showed that experimental EA in rats is associated with disorders largely correlating with those in patients with EA. This suggests that our experimental model can be used for development of new methods for the treatment of EA.

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REFERENCES

1. S. V. Petrov and N. T. Raikhlin, *Manual of Immunohistochemical Diagnosis of Human Tumors* [in Russian], Kazan (2000), P. 116-135.
2. A. G. Sabirov, M. V. Burmistrov, M. A. Mukhamedyarov, *et al.*, *Kazansk. Med. Zh.*, **90**, No. 2, 282-284 (2009).
3. V.-A. Yu. El'darkhanov, *Ter. Arkh.*, **69**, No. 2, 22-25 (1997).
4. S. Bruley des Varannes and C. Scarpignato, *Dig. Liver Dis.*, **33**, No. 3, 266-277 (2001).
5. I. Castagliuolo, P. Brun, M. Costantini, *et al.*, *J. Gastrointest. Surg.*, **8**, No. 1, 24-30 (2004).
6. R. De Giorgio, M. P. Di Simone, V. Stanghellini, *et al.*, *Am. J. Gastroenterol.*, **94**, No. 9, 2357-2362 (1999).
7. F. Farrokhi and M. F. Vaezi, *Orphanet. J. Rare Dis.*, **2**, 38 (2007).
8. J. R. Goldblum, R. I. Whyte, M. B. Orringer, and H. D. Appelman, *Am. J. Surg. Pathol.*, **18**, No. 4, 327-337 (1994).
9. S. Goto and J. L. Grosfeld, *J. Surg. Res.*, **47**, No. 2, 117-119 (1989).
10. A. Iqbal, B. Tierney, M. Haider, *et al.*, *Dis. Esophagus*, **19**, No. 3, 193-199 (2006).
11. J. Ivaska, H. M. Pallari, J. Nevo, and J. E. Eriksson, *Exp. Cell Res.*, **313**, No. 10, 2050-2062 (2007).
12. W. Park and M. F. Vaezi, *Am. J. Gastroenterol.*, **100**, No. 6, 1404-1414 (2005).
13. A. Sachdev, B. S. Sandhu, S. D'Cruz, *et al.*, *Indian J. Gastroenterol.*, **23**, No. 3, 109 (2004).
14. T. Takahashi, *J. Gastroenterol.*, **38**, No. 5, 421-430 (2003).