Abstract

Background/Aims: Esophageal motor abnormalities are frequently found in patients with gastroesophageal reflux disease. The effect of bile in esophageal dysmotility is probably secondary to mucosal signaling to the muscular layer and not a transmural process. This study aims to identify the mucosa-muscular signaling path by receptors blockade in an experimental study.

Methods: Fifteen guinea pig esophagi were isolated and *ex vivo* esophageal contractility was assessed with force transducers. The esophagi were incubated in 100 µM ursodeoxycholic acid for 1 hour and 5 sequential contractions induced by 40 mM KCl spaced by 5 minutes were measured. After 30 minutes, esophagi specimens were incubated in 3 different smooth-muscle contraction antagonists: atropine (1µM) in 5, suramin (1µM) in 5 and genistein (1µM) in 5. The same protocol for contractions was repeated. Values are expressed as mean ± standard deviation and encompass the mean of five stimuli. Experimental procedures were approved by the University Institutional Review Board.

Results: Contraction amplitudes after bile incubation but before antagonist incubation were 1.6±0.6 g, 1.2±0.8 g, and 1.2±0.4 g for atropine, suramin and genistein, respectively. Mean contraction amplitudes after antagonists instillation were 1.2±0.6 g, 1.4±0.5 g, 0.9±0.2 g, respectively. There was no different in contraction amplitude before and after instillation of atropine (p=0.188), suramin (p=0.488) or genistein (p=0.079).

Conclusion: Our results show that blockage of cholinergic (atropine), purinergic (suramin) or tyrosine kinase (genistein) paths do not change esophageal dysmotility induced by bile. Other molecular path may play the role in this scenario.
Introduction

Esophageal motor abnormalities are frequently found in patients with gastroesophageal reflux disease (GERD). This may be caused by acid and/or bile reflux. In a previous experimental study, esophageal exposure to ursodeoxycholic acid - a component of bile - decreased esophageal contraction amplitudes. This outcome was not observed when the esophageal mucosa was resected. These findings indicated that the effect of bile on esophageal motility is due to alterations of mucosal signaling to the muscular layer rather than a transmural process with direct muscular stimulation.

It is still elusive the molecular path responsible to this signaling. Since a myriad of paths are linked to smooth muscle contraction and relaxation, this study aims to try to identify the exact nature of the mucosa-muscular signaling path by receptors blockage from 3 different paths found in the esophageal muscle in an experimental study.

Materials and Methods

Animals

Fifteen guinea pigs (3 months, males, weight 200-250g) were studied. Animals were killed by spinal cord injury and cervical incision after 24 hours fasting. Esophagi were isolated, and its contractility assessed with force transducers.

Animals were randomly assigned into 3 groups:
Group A (n=5): ursodeoxycholic acid + atropine
Group B (n=5): ursodeoxycholic acid + suramine
Group C (n=5): ursodeoxycholic acid + genistein

Contraction experiments:

Contraction experiments followed previous protocol. Three-centimeter segments were obtained from the distal esophagus and were mounted in chambers for isolated organ perfusion containing Krebs-Henseleit solution oxygenated by a mixture of 95% O2 and 5% CO2 (pH 7.4). The segments were connected to force transducers (Soft & Solutions Ltda, São Paulo, Brazil) and attached to a micromanipulator to allow variation of basal tension. The specimens were kept with a basal tension of 1 g for 1 hour to stabilization after assembly. Developed force (contraction amplitude) was recorded.

The esophagi were incubated in 100 µM ursodeoxycholic acid for 1 hour and 5 sequential contractions induced by 40 mM KCl spaced by 5 minutes were recorded. Wash out was carried out between contractions. Then, esophagi specimens were incubated in 3 different smooth-muscle contraction antagonists: atropine (1µM), suramin (1µM) or genistein (1µM) according to groups division. After 30 minutes, the same protocol for contractions was repeated.

Statistics

Variables are expressed as mean ± standard deviation (range) and encompass the mean of 5 stimuli. Paired Student t test was used when appropriated since data followed a normal distribution according to the Kolmogorov–Smirnov test. A value of p was considered significant at the 0.05 level. A sample size calculation was performed based on the results of a previous study to allow an alpha error <0.05 and statistical power > 80%.

Ethics

The study was approved by the Institutional Review Board (#1390/11). There are no conflicts of interest. The authors are responsible for the manuscript and no professional or ghost writers were hired.

Results

Two (13%) esophagi specimens were unresponsive after 2 hours incubation. Their data was excluded and the experiment was repeated with new specimens for a total of 15.
Esophageal contraction amplitudes before and after incubation with antagonists are depicted in Table 1.

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<th>Before</th>
<th>After</th>
<th>p</th>
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<tbody>
<tr>
<td>Group A (atropin)</td>
<td>1.609 ± 0.628 (0.708 – 2.384)</td>
<td>1.232 ± 0.638 (0.686 – 2.252)</td>
<td>0.188</td>
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<tr>
<td>Group B (suramin)</td>
<td>1.173 ± 0.778 (0.528 – 2.464)</td>
<td>1.450 ± 0.462 (0.808 – 1.786)</td>
<td>0.448</td>
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<tr>
<td>Group C (genistein)</td>
<td>1.218 ± 0.406 (0.764 – 1.806)</td>
<td>0.913 ± 0.191 (0.676 – 1.130)</td>
<td>0.079</td>
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No difference in contraction amplitudes before and after incubation with antagonists were noticed for atropine (p=0.188, paired T test), suramin (p=0.449, paired T test) or genistein (p=0.079, paired T test).

**Discussion**

Bile duodeno-gastroesophageal reflux has been implicated in the genesis of GERD symptoms. However, the role of bile-induced esophageal dysmotility is still unclear. Only few studies focused on the effect of bile on esophageal motility, probably due to technical limitations for the detection of non-acid reflux. We hypothesized that an aggressive chemical stimulation of the mucosa may lead to a signaling to the muscular layer and a consequent abnormal peristalsis. Some indirect evidence supports this theory: (a) acute intraluminal infusion of HCl (Bernstein test) leads to GERD symptoms and changes in esophageal contraction amplitudes in humans; (b) the infusion of bile components also cause GERD symptoms; (c) the motility of ex vivo human esophagi strips is not altered by HCl incubation while the addition of the supernatant of mucosa layer bathed with HCl do alter motility; and (d) the removal of the mucosal layer of guinea pig esophagi abolishes bile-induced dysmotility.

**Methodology**

The objective of the current study was to evaluate changes in the amplitude of esophageal contraction of guinea pigs esophagi when exposed to different contraction antagonists to try identifying mucosa-muscular signalization pathways induced by ursodeoxycholic acid exposure: (a) atropine due to its anti-cholinergic effect, (b) suramin for its purinergic blockage and (c) genistein for its anti-tyrosine kinase activity.

The study protocol employed a careful methodology with isolated organ perfusion equipment based on the expertise from previous studies from the laboratory of the Department of Biophysics.

The same methodology was previously used as a model of bile-induced dysmotility that validated the use of: (a) guinea-pigs as the animal model; (b) KCl as the contraction inductor; (c) viability of isolated esophagi specimens; and (d) ursodeoxycholic acid as one component of bile. The results of this previous study were used to determine the ideal sample for the current experiment.

**Suramin**

Suramin is a molecule derived from the urea that has been used as an antimicrobial for the treatment of trypanosomiasis, HIV, hepatitis B, etc. It is also a P2-purinoceptor antagonist. P2X and P2Y purinceptors are the endpoint of the action of the adenosine triphosphate (ATP) acting as a cotransmitter with noradrenaline at the sympathetic neuromuscular
junction leading to smooth muscle contraction or relaxation.  

Imaeda et al. showed a decrease in the inhibitory junction potential of guinea-pigs esophagi incubated in atropine, electrically stimulated and exposed to suramin by blocking ATP. Cho et al. also showed a decrease in contractility of feline esophagi electrically stimulated and exposed to suramin. Our results did not show changes in contractility after exposure to suramin suggesting that the mucosa-muscular signalization is independent of ATP receptors.

Atropine
Atropine is a natural alkaloid found in some herbs, such as *Atropa belladonna* (belladonna or deadly nightshade) and *Datura stramonium* (Jimson weed or Devil's snare). It has a widespread use in medicine as an anticholinergic inducing cycloplegia, tachycardia, bronchial dilatation, xerostomy, and intestinal hypomotility. Atropin inhibits one or more phases of the smooth muscle contraction by its antimuscarinic action.

In humans, atropin leads to esophageal hypocontractility measured by manometry. Cao et al. also showed that feline esophagi have decreased motility when exposed to acid in an experimental model of acid esophagitis; however, contraction is unaltered when induced by acetylcholine stimulation. This experiment demonstrated, in accordance to our findings, that the cholinergic is not the signalization path for the muscular layer with either acid or biliary mucosal stimulation.

Genistein
Genistein, daidzein and glycitein are the main isoflavones – phytostrogens found in plants such as the soy. Genistein has been tried in the treatment of some hormone-dependent diseases such as some types of cancer, diabetes and menopause symptoms. Zhang et al. showed that genistein decrease murine gastrointestinal motility. This effect is not abolished by estrogen receptor blockade demonstrating a different path. In the same experiment, the authors blocked several other muscle receptors to show the effect of genistein on the gastrointestinal smooth muscle via α-adrenergic receptors, nitric oxide and cyclic adenosine monophosphate pathways, ATP-sensitive potassium channels, and inhibition of L-type Ca(2+) channels. Also, genistein activates tyrosine kinase contraction path.

Previous studies that focused on the action of genistein on the esophagus were conducted in isolated cultured cells only. Our results, similar to the other tested substances, did not support the paths inhibited by genistein as the mediators to bile-induced dysmotility.

Conclusions
The current experiment was limited to the study of the action of a single component of bile (ursodeoxycholic acid) in guinea-pig esophagi strips stimulated by chemical depolarization and incubated with a single dose of 3 different smooth muscle contraction antagonists. The control group to test the effects of Kcl and bile came from a previous publication. We considered unethical and unnecessary to sacrifice additional animals to repeat the same procedures previously performed. Our results showed, within these limitations, that blockage of cholinergic (atropine), purinergic (suramin) or tyrosine kinase (genistein) paths do not change bile-induced esophageal dysmotility. Other molecular path may play the role in this scenario. Its identification, in future studies, may allow pharmacologic treatment for patients with GERD esophageal dysmotility.

References
2. Vaezi MF, Singh S, Richter JE. Role of acid and


