Experimental study on the effect of bile, and bile and hydrochloric acid mixture on the esophageal mucosa

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Abstract
Introduction: The investigation of duodena gastro esophageal reflux (DGER) implies both clinical and experimental studies. Within the context of the literature, our study aimed to produce esophageal lesions by the development of an experimental model reproducing the characteristics of DGER and to analyze their microscopic pattern. Material and Methods: The material consisted in three groups of white Wistar rats. Group I (control group) included physiologic saline gavaged rats. Group II received by the esophageal probe bovine bile pH 7, and group III 0.5% bovine bile at pH 4, to which we added hydrochloric acid 0.1 N. The rats were sacrificed in the 21st day of the experiment. The esophagus was sectioned obliquely and longitudinally, maintaining the lumen and marking the upper and lower extremities. The esophageal fragments were routinely processed for light microscopy pathology exam, in HE staining. Results: The pathologic aspects suggested that the effect of bile and bile and hydrochloric acid mixture on the esophageal epithelium interferes with the normal keratinization process, with consequent onset of hyperkeratinization. Moreover, epithelial atrophy was present in the group II on restricted areas and in the group III on extended territories. Discussion: The alterations in the keratinization process suggest the possibility of initiation in time of carcinogenic mechanism. The atrophic transformations plead for an evolution towards erosion and ulceration. The study takes into consideration the differences between human and experimental animal esophageal epithelium. Conclusions: Thus, there emerge new perspectives to extrapolate the experimental results into the human biologic context, the morphologic pattern proving the irritant effect of DGER in vivo. Keywords: duodena gastro esophageal reflux, mucosa, bile, hydrochloric acid, experimental model.

Introduction
Although the cholecystectomy is considered a “successful” surgical procedure, an important percentage of the cholecystectomized patients (44% [1]) maintains the symptoms prior to the intervention or develops new ones. One of the causes of the postcholecystectomy syndrome is duodena gastro esophageal reflux (DGER) [1–5], investigated not only from the pathogenic and clinical features point of view but also from the perspective of long distance complications – following mainly the impact of the reflux on the esophageal mucosa [6–12].

There is very little information in the literature referring to the esophageal pathologic modifications, due to the difficulty (i) of in vivo dynamic investigation of the human esophagus subject to DGER; (ii) of the achievement of an animal model overlapping with physiopathology conditions specific to humans. Although successfully reproducing esophagitis, Barrett’s esophagus and esophageal adenocarcinoma the animal models [6, 13–24] cannot separate the individual components of the refluxate, such as acid or bile [23], are incapable of controlling the amount and concentration of the refluxate and determine significant postoperative stress and morbidity [23]. The new trend in the investigations on this subject is to design and implement new experimental models [22–24], the animal data suggesting synergistic damaging effects for both bile and acid in esophageal mucosal injury, concurring with preliminary human studies.

Within this framework, the developed experimental study completes the data existing in the publication mainstream, our results supporting the hypothesis according to which prolonged excessive contact between esophageal mucosa and bile or bile and acid mixture determines esophageal lesions.

Material and Methods

Description of the experimental model
There were investigated three groups of white Wistar rats with an average weight of 350 g, all males. The rats were kept for two weeks before the beginning of the experiments in standard laboratory conditions, in rooms with an average temperature of 22°C, relative humidity (55% ± 5%) and normal circadian rhythm. The research respected the international regulations on the work with experimental animals.
The administration of the substances was performed for 21 days, at a 12 hours distance (8.00, 20.00), by gavage through an esophageal probe introduced up into the level of the inferior half of the esophagus. The approximate length of the esophagus is 4 cm and the probe was introduced for 3 cm. We chose this method instead of the surgical model in order to avoid post-operator malnourishment and the surgical stress on the experimental animals.

The control group (group I) included five rats gavaged with physiological saline. The second group formed by six rats received through the esophageal probe 0.5% bovine bile (SIGMA), pH 7, kept at 4°C. The quantity necessary for the daily treatment was kept at room temperature with at least a half an hour before the administration. Every time, the quantity administered was 1 ml solution/100 g body weight.

The third group (lot III) included 12 rats receiving 0.5% bovine bile at pH 4, to which we added hydrochloric acid 0.1 N. The solution was kept in the same conditions as described above and identically administered. The addition of hydrochloric acid in the bovine bile was intended to experimentally create DGER.

There was kept the same food intake for all the duration of the experiment.

The rats were sacrificed in the 21st day of the experiment. The esophagus was sectioned obliquely and longitudinally, maintaining the lumen and marking the upper and lower extremities. Thus, we were able to investigate in seriated sections the modifications occurred on the entire length of the esophagus and comparatively on the walls and at the extremities.

Pathology exam

The esophageal fragments were routinely processed for light microscopy pathology exam, in HE staining.

Results

Control group: simple gavage

We identified a thin stratified squamous keratinized epithelium, with the same features in all experimental animals. Thus, stratum spinosum was formed by 2–5 cell sheets, stratum granulosum presented 1–2 cell sheets continuously disposed and stratum corneum had the equivalent thickness of maximum five overposed sheets of squamous cells with a compact aspect (Figures 1 and 2).

In the pathologic investigation of the esophageal seriated sections belonging to the five experimental animals, we identified some isolated particular aspects:

- a small area, situated on an esophageal wall where the epithelium had a reduced thickness, with a stratum spinosum formed by a single cell sheet and a thin stratum corneum, corresponding to 1–2 squamous cell sheets containing keratin, detaching in multiple strips;
- a small area at the proximal esophageal extremity where the epithelium was thicker, the stratum spinosum having 5–6 cell sheets and stratum corneum 8–10 cells loaded with keratin, either compactly disposed, or detaching in strips;
- five isolated areas situated on the esophageal walls where the epithelium presented hyperacanthosis – stratum spinosum having over 10 sheets of polyhedral cells in one of the areas and over 20 cell sheets in the other four areas; stratum granulosum and stratum corneum had a similar thickness with the adjacent areas.

Lamina propria had the aspect of a dense connective tissue with conspicuous collagenization; the presence of monocytes, lymphocytes and macrophages was appreciated as a moderate inflammatory infiltrate.

The submucosa and muscularis mucosae presented no pathologic modifications.

Group II: bile gavage

The stratified squamous keratinized epithelium had a heterogenous morphology, alternating along either the esophageal walls or differing from one wall to the other (Figures 3 and 4). These aspects were mainly due to the organization manner of stratum spinosum and stratum corneum. Stratum spinosum had on large areas a thickness of only 1–2, maximum 3 cell sheets, giving to the epithelium an atrophic aspect. Nevertheless, we observed areas where stratum spinosum consisted of 5–6 cell sheets, and at one of the experimental animals we encountered areas with as many as 10 sheets of polyhedral cells. Stratum corneum presented a normal thickness alternating with reduced thickness or hyperkeratinization. The differences in the thickness of the keratin could not be correlated with the thickness of stratum spinosum. Stratum granulosum was continuous and, at two experimental animals, we could also identify the stratum lucidum, with cells charged with eleidin.

A particular aspect observed in a single experimental animal was the existence of intraepithelial vesicles with consequent detaching towards the lumen of the overlying epithelium – this type of lesion might suggest a beginning of erosive esophagitis.

In five from the six experimental animals, the lamina propria was intensely collagenic, giving a fibrous aspect; in two of the experimental animals we observed a well represented chronic inflammatory infiltrate. One of the six experimental animals maintained the features of loose connective tissue in the lamina propria, where we also noted edema.

Group III: bile and hydrochloric acid gavage

The stratified squamous keratinized epithelium presented different morphologic aspects due to the organization manner of stratum spinosum and stratum corneum (Figures 5 and 6).

In five animals from this group, 80–90% of the esophageal surface was lined with a very thin epithelium, with a single sheet of flat cells in stratum spinosum, a discontinuous stratum granulosum and a thin stratum corneum, often detached.

In four animals, the epithelium presented variable thickness throughout the length of the same wall, or on one wall as compared with the other. These differences appeared because of the changes in the number of cell sheets of stratum spinosum (1–2 or 3–5); the overlying stratum corneum had a thickness correlated with the one of stratum spinosum.
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Consequently, in the areas where stratum corneum was very well represented we observed territories where the two walls were united by keratin. At two animals, the epithelium had a stratum spinosum with 2–3 cell sheets and a very thick stratum corneum. In one of these animals there was an important dissociation between stratum spinosum and stratum corneum, the latter having a homogenous aspect, intensely acidophil, astructural, suggesting a transformation process toward fibrin necrosis.
At the other animal, the keratin on one wall presented on extensive area coalescence with the keratin from the contra lateral wall. One of the animals presented necrotic esophagitis, the epithelium (as well as the entire mucosa) being replaced by a fibrin cellular exudate.

Although stratum spinosum and stratum corneum had different aspects, the stratum basal cells presented in the entire group (except for the necrotic esophagitis case) extremely intense basophilic nuclei suggesting an important proliferative/regenerative activity.

The lamina propria had an intensely collagenic aspect, sometimes with conspicuous inflammatory cell elements. The sub mucosa and the muscularis presented no pathologic modifications.

Discussion

The researchers’ preoccupations for the esophageal lesions investigated on experimental models focus on different pathologic entities.

For the reflux esophagitis there were investigated the pathologic modifications, cell proliferation, apoptosis and oxidative stress [16, 19, 22–24]. According to recent studies [22, 23], the bile and, respectively, acid perfusion determined the presence of the inflammatory cells in mucosa, hyperproliferation of basal cells, papillae hypertrophy and hyperkeratinization, associated with extensive epithelial sloughing and ulceration (some of them involving nearly 50 to 90% of the mucosal thickness). However, bile-perfused esophagus had more severe epithelial ulceration than the acid-perfused esophagus [23]. In contrast to bile perfusion, no pathologic modification occurred in the esophagus of the saline perfusion animals [22]. Similar results are reported in [24], referring to the role of pancreatic trypsin in chronic esophagitis induces by GDER: erosions and ulcer formation as well as marked thickening of the esophageal wall.

Other studies investigated Barrett esophagus and carcinogenesis process for the esophageal adenocarcinoma [13–18, 20, 21]. The experimental data as well as the results obtained on human subjects [9] lead to the conclusion that the influence of bile reflux on the development of esophageal adenocarcinoma needs to be studied more before establishing causality.

The morphologic landscape characterizing the esophageal mucosa permitted, by comparing the results from the three lots, an assessment of the action of bile and acid associated with hydrochloric acid, respectively, on this mucosa.

The first group, subjected to simple gavage presented a stratified squamous keratinized epithelium with constant features: stratum spinosum formed by 2–5 cell sheets, stratum granulosum with 1–2 cell sheets with continuous arrangement and stratum corneum with an equivalent thickness of five overlapping squamous cell sheets, with a homogenous aspect.

The isolated aspect of detaching in strips observed in the stratum corneum as well as the presence of keratin in the gastric lumen are elements confirming the irritant mechanic action of the gavage itself.

The second group, gavaged with bile, was generally characterized by a stratified squamous keratinized epithelium which, compared to the first group, had a heterogeneous morphology, the atrophic territories alternating with the hypertrophic ones. The layers responsible for the new morphologic configuration were stratum spinosum and stratum corneum with a conspicuous tendency towards hyperkeratinization, even in the areas with a thinner stratum spinosum. Consequently, the hyperkeratinization could not be correlated with the thickness of stratum spinosum. The presence of intraepithelial vesicles, with the subsequent detaching of the overlying epithelium towards the lumen was an important element supporting the erosive esophagitis lesions.

The microscopic aspects suggest that the action of bile on the esophageal epithelium affected the normal keratinization process, mainly in the cells of stratum spinosum, which changed very rapidly, on fewer cell sheets, charging with keratin and forming a well represented stratum corneum, hence the character of hyperkeratinized epithelium.

The third group, gavaged with bile and hydrochloric acid revealed – as the second group – heterogeneous morphologic characteristics also due to stratum spinosum and stratum corneum.

Unlike the second group, the general aspect was that of atrophic epithelium, by the significant reduction of the number of cell sheets in stratum spinosum and stratum corneum; isolated hyperkeratinized areas were also present.

These microscopic aspects support the effect of bile and hydrochloric acid on esophageal epithelium, resulting in the perturbation of the normal keratinization process, the keratin-loaded cells from stratum corneum being injured by the erosive action of hydrochloric acid. In order to compensate/support the epithelial regeneration demanded by the disruptions in the superficial stratum corneum, the basal cells presented intensely basophilic nuclei, indicating an important proliferative/regenerative process.

The experimental model is valuable by the modifications developed in the esophageal mucosa, under the effect of bile and bile and hydrochloric acid mixture. Our results draw the attention over the interferences between the administered substances and the esophageal epithelium (stratified squamous keratinized), leading to alterations in the histoarchitectonic of the cell layers and proving the perturbations in the keratinization process. Nevertheless, we must stress the fact that the human esophageal epithelium is of the stratified squamous non-keratinized type, thus less resilient than that of the animals utilized in this experiment.

Human esophageal epithelial histology includes (1) a basal stratum consisting from one sheet of cuboidal cells, with proliferative purpose, (2) a stratum spinosum formed by 3–6 polyhedral cell sheets, joined by desmosomal junctions having in their cytoplasm tonofilaments of cytokeratin and cell organs involved in keratin synthesis; however, the keratinisation process is genetically controlled and keratin does not form in
this location; (3) a superficial layer, formed by several overgrown squamous cell sheets, maintain their nuclei. The lack of superficial keratin makes the human esophageal epithelium much more sensitive to the damaging effect of the bile and/or the bile associated with hydrochloric acid. Consequently, in humans and in vivo, the normal histarchitectonic status can be affected resulting morphologic transformations in the spinous or superficial layer.

These transformations, either proliferative (under the effect of bile) or atrophic (under the effect of bile and hydrochloric acid) represent the reaction modality of the epithelial cells.

In the first instance, cell proliferation can be considered as a first stage in a hyperplasia–dysplasia–neoplasia type sequence, thus supporting the possibility of the development in time of a malignant transformation within the context of biliary reflux.

In the second instance, the atrophy can be interpreted as a first modification in the evolution towards erosion–ulceration, thus supporting the possibility of the development in time of ulcer lesions within the context of acid reflux.

The differences that occurred can be explained by the design of the experiment. Unlike the experimental models applied by [22, 23], based on external esophageal perfusion, achieved via a micro-pump ensuring a constant flow over a long period of time, we performed a bile/bile and hydrochloric acid administration through gavage, implicitly having a discontinuous and transitory character. Consequent to this discontinuous/transitory exposure, the esophageal mucosa reacted through the development of an important epithelial atrophy.

Conclusions

Our experimental model allowed the identification of specific morphologic lesions. The proliferative transformations support, within the context of hyperplasia–dysplasia–neoplasia sequence, the possibilities of initiation in time of the carcinogenic mechanism. The atrophic transformations plead for an evolution towards erosion and ulceration. Thus, several perspectives of extrapolation towards the human biological context obtained morphologic landscape proving the irritant effect of biliary reflux in vivo.

References


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