A weakly acidic solution containing deoxycholic acid induces esophageal epithelial apoptosis and impairs integrity in an \textit{in vivo} perfusion rabbit model

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**ABSTRACT**

**Introduction:** Impaired esophageal mucosal integrity may be an important contributor in the pathophysiology of GERD. Nevertheless, the effect of potentially harmful agents on epithelial integrity is mainly evaluated *in vitro*, for a short period of time and the possible induction of epithelial apoptosis has been neglected.

**Objective:** To assess the effect of an acidic and weakly acidic solution containing DCA on the esophageal epithelium in an *in vivo* rabbit model of esophageal perfusion and evaluate the role of the epithelial apoptosis.

**Methods:** The esophagus of 55 anesthetized rabbits was perfused for 30 minutes with different solutions at pH 7.2, pH 5.0, pH 1.0 and pH 5.0 + 200 and 500 µM deoxycholic acid (DCA). Thereafter, animals were sacrificed immediately, at 24 or 48 hours after the perfusion. Transepithelial electrical resistance (TEER), epithelial dilated intercellular spaces (DIS) and apoptosis were assessed in Ussing chambers, transmission electron microscopy and TUNEL staining, respectively.

**Results:** No macroscopic or major microscopic alterations were observed after the esophageal perfusions. The acidic and weakly acidic solution containing DCA induced similar long lasting functional impairment of the epithelial integrity but different ultrastructural morphological changes. Only the solution containing DCA induced epithelial apoptosis *in vivo* and *in vitro* in rabbit and human tissue.

**Conclusion:** In contrast to acid, a weakly acidic solution containing DCA induces epithelial apoptosis and a long-lasting impaired mucosal integrity. The presence of apoptotic cells in the esophageal epithelium may be used as a marker of impaired integrity and/or bile reflux exposure.
INTRODUCTION

With 20% of the population in Western countries complaining of gastro-esophageal reflux disease (GERD) symptoms at least once weekly (5; 23) and a globally rising trend, GERD is a highly prevalent disorder. In the spectrum of disorders grouped as GERD, non-erosive reflux disease (NERD) is the most common phenotype, covering 60% of all the people suffering from typical reflux symptoms. The fact that separates them from the rest of the group is that they do not show lesions during routine endoscopic examination. (24; 38) Up to 40% of the GERD patients do not completely respond to PPI therapy, which may indicate that other factors apart from acid are responsible for symptom generation. Using combined 24h pH and duodenogastro-esophageal reflux (DGER) monitoring, Tack et al. showed in refractory GERD patients that symptom episodes can be attributed to DGER and that 40% of NERD patients have increased bile reflux (34). It has also been shown that patients receiving PPI therapy have an increased concentration of unconjugated bile acids due to the weakly acidic environment and gastric bacterial overgrowth. (35) In vitro studies show that the bile acid deoxycholic acid impairs esophageal epithelial integrity (32) and is genotoxic in epithelial cells in Barrett’s esophagus. (14) Similar experiments in rabbit and human esophageal tissue showed a pronounced effect of DCA on the functional integrity of the tissue expressed by a significant drop in transepithelial electrical resistance (TEER) (2; 9). A study by Zhang et al. (43) showed that some bile acids, including DCA, have the ability to cause apoptosis of human normal esophageal epithelial cells in weakly acidic conditions. In contrast, the presence of increased apoptotic epithelial cells in the esophageal mucosa of GERD patients has been neglected. Only one study showed that apoptosis in the squamous epithelium is increased in parallel with the severity of the esophagitis and is decreased after anti-reflux surgery. (40) The luminal agents of the
refluxate responsible for the induction of apoptosis are unknown and bile acids, particularly DCA, may be plausible candidates. To our knowledge, the effects of DCA on the integrity of esophageal epithelium were never evaluated in vivo. In contrast to DCA, the effect of acid on the esophageal epithelium in vivo has been extensively studied.(6; 15; 17; 20; 22; 28) Esophageal acid perfusion impairs mucosal integrity, induces dilated intercellular spaces (DIS), and provokes cell necrosis in the long term. Nevertheless, the long lasting effect of acid on mucosal alterations has never been evaluated.

Impaired mucosal integrity is obviously present in GERD patients with erosions but also in the non-eroded areas of GERD patients,(18) in some patients with NERD (6) and in refractory GERD patients with absence of erosions at endoscopy.(29) The general believe is that the acidity of the gastric refluxate is responsible for both symptoms and damage of the epithelium. Nevertheless, it is unknown whether the presence of an impaired integrity is due to a continuous chronic caustic effect of the reflux events or to a slow and/or incomplete long lasting effect of some reflux episodes that occurred earlier (several hours or days) and provoke severe impaired integrity. Based on the first scenario, one should expect an association between some reflux parameter measured with the multichannel intraluminal impedance (MII)-pH technique and the status of the mucosa. Nevertheless, in adults, only a moderate correlation was reported between acid exposure time and mucosal integrity assessed by baseline impedance.(6; 7; 19; 30) This can be explained by the fact that mucosal integrity may depend not only of an acute rapid effect of reflux events, but also on long lasting changes provoked by severe events that occurred hours or even days before. However, this hypothesis is very difficult to prove in humans but also in animals, since all currently available reflux models for the study of epithelial integrity, do not allow recovery
of the animal after esophageal perfusion. That is why we recently developed a rabbit model of esophageal perfusion in which the animal can be studied several days after perfusion.(6)

We hypothesize that luminal acid and DCA may alter in vivo the esophageal epithelium, leading to structural abnormalities, which result in long lasting disruption of the epithelial barrier. Hence, the aims of the present study are to evaluate in an in vivo rabbit model of esophageal perfusion 1) whether acidic and weakly acidic solutions containing DCA can impair mucosal integrity, 2) whether changes in integrity are associated with the induction of apoptosis and finally 3) to investigate the long lasting effect of these solutions on mucosal integrity. Then, some of the findings will be evaluated in vitro in rabbit and human tissue.

METHODS

Animals

Fifty-five adult New Zealand White rabbits weighing 2.5-4 kg were used. They were anaesthetized with intramuscular ketamine chloride (40 mg.kg\(^{-1}\)) and xylazine (10 mg.kg\(^{-1}\)) (Parke-Davis, NV Warner-Lambert, Zaventem, Belgium). Esophageal perfusion was performed as described previously.(6) Briefly, a polyvinyl tube was attached to an impedance catheter of 1.5mm diameter (prototype by Professor Jiri Silny, University Hospital Aachen, Germany) between the second and the third impedance channel, 8 cm proximal to the lower impedance channel. The assembly used had three recording impedance segments over a distance of 14 cm spaced at 3 cm intervals. Then, it was passed orally into the esophagus and the first sensor was located 2cm above the lower esophageal sphincter (LES). Impedance readings were used to localize the esophagus and to monitor the administration of the perfusate. Perfusion was performed at 1 ml.min\(^{-1}\) allowing perfusion of the lower two impedance recording segments (10cm above the LES) for 30 minutes. This
catheter configuration and the perfusion flow rate allow to perfuse two thirds of the esophagus avoiding the aspiration into the lungs as seen by a stable impedance baseline of the more upper channel (channel 3) during the perfusion. In contrast, impedance baseline of the 2 distal channels dropped few seconds after initiating the perfusion. During the perfusions, sedation of the rabbits was maintained with ketamine. All solutions were perfused at room temperature. When the perfusion period was finished, the impedance catheter was removed and the animals were allowed to recuperate for 40 minutes and then sacrificed (0h group) by a blow on the neck followed immediately by exsanguination. A second cohort of animals was sacrificed at 24h (24h group) and a third cohort at 48h (48h group) after the perfusion. The esophagus was excised in its entirety, stripped of its muscle layers and opened in a paraffin tray containing carbogenated Krebs-Henseleit bicarbonate buffer (KHBB, pH 7.4 containing (in mM): 118 NaCl; 4.7 KCl; 1.2 CaCl2; 1.2 MgSO4; 1.2 NaH2PO4; 25 NaHCO3 and 11 glucose). Solutions used in the perfusion were the following:

(1) neutral solution: 0.9% NaCl at pH 7.2; (2) weakly acidic solution at pH 5.0: 0.9% NaCl containing pepsin (1mg.ml⁻¹); (3) weakly acidic solution at pH 5.0: 0.9% NaCl containing pepsin (1mg.ml⁻¹) and 0.2mM DCA; (4) weakly acidic solution at pH 5.0: 0.9% NaCl containing pepsin (1mg.ml⁻¹) and 0.5mM DCA; and (5) acidic solution at pH 1.0: 0.9% NaCl. Two other cohorts of animals were sacrificed 24h or 48h after perfusion with weakly acidic solution at pH 5.0: 0.9% NaCl containing pepsin (1mg.ml⁻¹) and 0.5mM DCA and acidic solution at pH 1.0: 0.9% NaCl.

**Transepithelial electrical resistance**

4 distal esophageal mucosal sections were cut and mounted in Ussing chambers (Mussler Scientific Instruments, Aachen, Germany) with an aperture of 0.5 cm². These sections were obtained from the area exposed to the perfused solution, exposure to the experimental
solution was confirmed by impedance monitoring, as mentioned above. The tissue was incubated in 37°C carbogenated KHBB and transmucosal potential difference (PD) was continuously monitored with Ag/AgCl electrodes. Basal TEER was calculated according to Ohm's law from the voltage deflections induced by bipolar constant current pulses of 50 µA (every 6s) with duration of 200ms applied through platinum wires (Mussler Scientific Instruments, Aachen, Germany). Fluid resistance was taken into account and corrected for. The average of 4 tissues was used to calculate the TEER and PD in each animal.

**Morphological studies**

**Tissue preparation**

Following the TEER experiments in Ussing chambers, tissues were examined using both light microscopy and transmission electron microscopy (TEM) to assess the integrity and morphology of the tissue as described before. One of the 4 tissues was fixed in 2.5% (w/v) glutaraldehyde in phosphate buffer for TEM and two others were fixed in 4% (w/v) paraformaldehyde for conventional light microscopy and TUNEL (Terminal deoxynucleotidyl Transferase mediated dUTP Nick-End Labeling) staining, respectively. Light microscopy was performed with the tissue embedded in paraffin. Transverse sections (5 mm) were stained using haematoxylin–eosin and von Gieson methods and analyzed in a blinded fashion by an expert pathologist (MV).

**Measurement of DIS**

4 TEM microphotographs per animal were taken (x4000 magnification) and analysed in a blinded fashion using custom-written image analysis software in IGOR Pro (WaveMetrics Inc., Oregon, USA). Intercellular spaces were delineated between 5 and 10 epithelial cells from the basal and lower prickle layers in each microphotograph. The intercellular spaces area was measured and related to the perimeter of the corresponding cells to obtain a
relative measure of DIS. The morphological evaluations were performed by two investigators (MV, RF) who were blinded to the type of mucosal exposure (test solution).

In Situ detection of DNA fragmentation
Frozen sections were subjected to TUNEL staining and analyzed in a blinded fashion to detect in situ genome fragmentation and for confirming apoptosis. End labeling of exposed 3’ OH ends of DNA fragments was undertaken with the TUNEL in situ cell death detection kit AP (Roche Diagnostics) as described by the manufacturer. Nuclei were stained by incubation of the sections with DAPI. Two photographs at x20 magnification were taken in each animal and the percentage of apoptotic cells was quantified by dividing the amount of cell layers with apoptotic cells in a specific region by the total amount of cell layers of the epithelium. The number of cell layers was measured by counting the nuclei. A total of 3 measurements were taken per photograph.

In vitro exposure of esophageal tissue to DCA
Rabbit tissue
Esophageal mucosal sections of three additional rabbits were cut and mounted in Ussing chambers as described earlier.(9) After 1h of stabilization of the tissue KHBB was replaced with KHBB at pH 5.0 containing pepsin (1mg.ml⁻¹) as a control or KHBB at pH 5.0 containing pepsin (1mg.ml⁻¹) and 1mM DCA. After 2h of exposure, the experimental solution was replaced with KHBB pH 7.4 and tissue was allowed to recuperate for 1h. Tissue exposed to the control solution was removed and fixed at that point in time (4h after mounting). Tissues exposed to DCA were removed from the Ussing chambers at different time points: after 1h stabilization (time 0 minutes), at 30, 60, 90, 120 minutes of exposure and 60 minutes after
ending exposure. After removal, tissues were fixed in paraformaldehyde and prepared for TUNEL staining.

Human tissue

The entire esophagus was procured by the same surgeon (LC) from two organ donors following brain-death (DBB; 51 and 54 years old, both male) and one donor after cardiac-death (DCD; 56 years old, male). Organs were perfused with University of Wisconsin solution (Belzer UW®, Bridge to Life, Columbia, SC, USA) and stored/transported on ice for 105, 154, 163 minutes after which the esophageal epithelium was isolated by gentle dissection. Then, the epithelium was exposed *in vitro* with the same solutions and in the same manner as described above for the rabbit tissues. They were removed from the chambers at the end of the experiment and prepared for TUNEL staining. The percentage of apoptotic cells was quantified as described before.

**Statistics**

All data are presented as mean±SEM. Comparisons between groups were analysed using one-way ANOVA or Kruskal-Wallis test when appropriate. Then, when these tests were significant, Bonferroni's or Dunn's multiple comparison test was used to determine the group/s with statistical significant difference. The effect of the different solutions on time-TEER curves was analysed using two-way repeated measures ANOVA. When the ANOVA test was significant, Bonferroni's test was used to determine the times with statistical significant difference. Pearson’s *r* or Spearman’s *ρ* were used to determine correlations when appropriate. For calculating TEER, four tissues were averaged per animal. For calculating DIS, four tissues were averaged per animal and three microphotographs were averaged per tissue. Significance was declared at *p*<0.05.
RESULTS

Acute effect of in vivo acid and weakly acidic perfusion on epithelial integrity

Functional evaluation

TEER of the esophageal epithelium perfused with the control solution was 2209±77 Ω x cm². After perfusion with the acidic solution at pH 1.0, TEER was reduced by approximately 70% (434±79 Ω x cm², p<0.01). Perfusion with the weakly acidic solution at pH 5.0 did not affect TEER (2420±161 Ω x cm²). In contrast, the weakly acidic solution containing the unconjugated bile acid DCA, concentration dependently reduced TEER reaching an effect at 500 µM (p<0.01) comparable to the acidic solution at pH 1.0 (figure 1 A).

In a similar manner, the PD was reduced by DCA (p<0.01) and the acidic solution at pH 1.0 (p<0.05) (figure 1 B). Moreover, we also found a highly significant correlation between PD and TEER (r=0.92, N=32, p<0.0001) with the different test solutions assessed, indicating that at least in the rabbit esophagus and after acute exposure, PD is a good marker to estimate the status of mucosal integrity (figure 2).

Histological evaluation

None of the perfusions showed erosions or any histological abnormality when examined through light microscopy (data not shown). After the perfusion with the different solutions, the thickness of the epithelium, the thickness of the basal cell layer and the length of the papillae were unchanged compared with the control solution at pH 7.2 (table 1).

Table 1. Histological evaluation of the esophageal epithelium after the perfusion of the acidic and weakly acidic solution containing DCA.

<table>
<thead>
<tr>
<th></th>
<th>pH 7.2</th>
<th>pH 5.0</th>
<th>pH 5.0 +DCA 500 µm</th>
<th>pH 1.0</th>
<th>ANOVA p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial thickness (µm)</td>
<td>163.2±27.6</td>
<td>156.7±33.2</td>
<td>175.3±31.5</td>
<td>169.5±16.1</td>
<td>0.97</td>
</tr>
<tr>
<td>Basal cell layer (%)</td>
<td>14.22±0.9</td>
<td>13.6±3.7</td>
<td>13.4±2.5</td>
<td>12.8±1.2</td>
<td>0.93</td>
</tr>
<tr>
<td>Papillae length (%)</td>
<td>29.8±5.9</td>
<td>48.8±9.2</td>
<td>37.7±16.9</td>
<td>39.7±7.2</td>
<td>0.60</td>
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</table>
Thickness of the basal cell layer and length of the papillae were calculated as the % of the total epithelial thickness. Data are mean±SEM; n=4-7 animals per group. DCA: deoxycholic acid.

Nevertheless, at the ultrastructural level, the solution at pH 1.0 provoked widening of the intercellular space in the basal (from 0.04 ± 0.02 µm to 0.56 ± 0.13 µm, N=4, p<0.05) and in the prickle cell layers (from 0.14 ± 0.02 µm to 0.50 ± 0.04 µm, N=4, p<0.05) (figure 3C and G) when compared with the control solution (figure 3A and E).

Surprisingly, although the weakly acidic solution at pH 5.0 did not change TEER, an increase in the intercellular space from 0.04 ± 0.02 µm to 0.20 ± 0.01 µm (N=4, p<0.05) was observed only in the basal cell layer of the epithelium (figure 3B, F). In addition, the weakly acidic solution containing 500 µM of DCA also induced DIS in the basal cell layer 0.29 ± 0.03 µm (N=4, p<0.05, figure 3D). In the prickle and more superficial cell layers, intercellular spaces could not be measured since the interdigitations of the epithelial cells were lost and the epithelial morphology was altered. Moreover, ultrastructural abnormalities compatible with apoptosis (12) like margination and condensation of chromatin, intact cell membrane, and loss of electron-density (empty cytoplasm) were observed (figure 3H). In contrast, findings compatible with apoptosis were not observed after perfusion with the acidic solution at pH 1.0 (figure 3G). TUNEL staining further confirmed the presence of apoptotic cells in the superficial and the prickle cell layers of the epithelium only in animals perfused with the solution containing DCA (3-4 animals per condition, figure 4A and C). In animals perfused with the acidic solution, apoptotic cells were only found in the most upper functional layer (figure 4B). The quantification of TUNEL staining shows that animals perfused with DCA have more apoptotic cells in the epithelium (52.4±1.3 % vs. 10.7±0.8, p<0.01). Similar to the
solution at pH 5.0, animals perfused with the acidic solution show apoptotic cells only in the
most superficial cell layers of the epithelium reaching the 17.4±1.3 % of the total epithelium.

**Long lasting effect of in vivo acid and weakly acidic perfusion on epithelial integrity**

*Functional evaluation*

24h after the perfusion with pH 1.0, TEER recovered but was still lower compared to the
control solution pH 7.2 (1660 ± 123 Ω x cm² vs. 2209 ± 77 Ω x cm², p<0.05). The recovery was
completed after 48h as is shown by a TEER of 2055 ± 251 Ω x cm² (figure 5A).

In contrast to the acidic solution, 48h after perfusion with 500µM DCA the TEER still
remained lower compared to the control solution at pH 5.0 (1780 ± 128 Ω x cm² vs. 2421 ±
161 Ω x cm², p<0.05) despite the gradual increase of TEER after 24h and 48h (figure 5B).

The PD was recovered 24h after the perfusion with the acidic solution at pH 1.0 and the
weakly acidic solution containing 500µM DCA (figure 5C and D) despite that TEER was still
reduced. In contrast to the experiments with the acute exposure there is no correlation
between PD and TEER (r=-0.19, p=0.4) 24h and 48h after the perfusion with the different
test solutions, indicating that in the presence of a long lasting impaired epithelial integrity,
PD is not a good parameter to estimate changes in esophageal integrity.

*Histological evaluation*

None of the perfusions showed erosions or any histological abnormality when examined
through light microscopy (data not shown). The thickness of the epithelium was altered after
the esophageal acid perfusion (ANOVA p<0.05) but the post hoc analysis did not identify
statistical significance at any of the time points evaluated. The thickness of the basal cell
layer and the length of the papillae were unchanged in all conditions (table 2).
Table 2. Histological evaluation of the esophageal epithelium 24 and 48 hours after the perfusion of the acidic and weakly acidic solution containing DCA.

<table>
<thead>
<tr>
<th>pH 1.0</th>
<th>t=0h</th>
<th>t=24h</th>
<th>t=48h</th>
<th>ANOVA p value</th>
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<tr>
<td>Epithelial thickness (µm)</td>
<td>169.5±16.1</td>
<td>148.0±15.0</td>
<td>218.4±16.2</td>
<td>0.047</td>
</tr>
<tr>
<td>Basal cell layer (%)</td>
<td>12.8±1.2</td>
<td>12.1±0.8</td>
<td>12.7±1.8</td>
<td>0.98</td>
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<tr>
<td>Papillae length (%)</td>
<td>39.7±7.2</td>
<td>30.2±2.3</td>
<td>26.5±3.8</td>
<td>0.37</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH 5.0+DCA 500 µm</th>
<th>t=0h</th>
<th>t=24h</th>
<th>t=48h</th>
<th>ANOVA p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial thickness (µm)</td>
<td>175.3±31.5</td>
<td>147.2±20.7</td>
<td>195.3±57.2</td>
<td>0.78</td>
</tr>
<tr>
<td>Basal cell layer (%)</td>
<td>13.4±2.5</td>
<td>18.2±3.1</td>
<td>10.0±5.0</td>
<td>0.31</td>
</tr>
<tr>
<td>Papillae length (%)</td>
<td>37.7±16.9</td>
<td>29.9±6.5</td>
<td>21.1±5.6</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Thickness of the basal cell layer and length of the papillae were calculated as the % of the total epithelial thickness. Data are mean±SEM; n=4-7 animals per group. DCA: deoxycholic acid.

At the ultrastructural level, dilated intercellular spaces remained present 24h after perfusion with the acidic solution (figure 6B) although the magnitude was reduced from 0.57±0.13 µm to 0.14±0.03 µm (p<0.05; N=4). 48h after perfusion, the esophageal epithelium had a normal morphology and DIS were not present anymore (figure 6C). 24h after the perfusion with the weakly acidic solution containing 500µM DCA, a loss of normal cell morphology in the basal layer (figure 6E) was observed and the intercellular spaces could not be measured at this time point. Nevertheless, cells of the prickle and the functional cell layers showed an apparent normal morphology. After 48h, the epithelial morphology in the basal cell layer largely recovered and enlarged intercellular spaces were observed (0.33±0.04µm; p<0.05; N=4, figure 6F).

*In vitro* exposure of the esophageal epithelium to DCA
To elucidate whether DCA is able to impair mucosal integrity and to induce apoptosis in the esophageal epithelium *in vitro*, we exposed human and rabbit tissues in Ussing chambers to evaluate TEER and the presence of apoptotic cells. To evaluate the onset of apoptosis we evaluated the exposed tissue at different time points. When the epithelium was exposed for the initial 30 minutes to the control solution at pH 5.0, TEER was 2184±92 Ω x cm². In contrast, 30 minutes after exposure to DCA 1 mM, TEER significantly dropped to 1308±204 Ω x cm² (N=3, p<0.05) and remained significantly lower for 2h when compared with the control solution (N=3, p<0.001) (figure 7A). After examining the rabbit epithelium at each time point after TUNEL staining, we observed that apoptosis started to occur 90 minutes after the incubation with the weakly acidic solution containing 1mM DCA (ANOVA p=0.015) and was effecting epithelial cells from the superficial and the prickle cell layers (figure 7C and D). At this time point, 54.3±4.9 % of the epithelium was apoptotic but only an 8.9± 1.7 % of the epithelium showed apoptosis when the tissue was exposed to the control solution at pH 5.0 (p<0.05). More interesting was that similar results were reproduced in human tissue exposed to the same solutions and conditions. TEER was reduced by 1mM DCA (ANOVA p<0.05) reaching statistical significance at 120 and 150 minutes. Moreover, at the end of the exposure period with the solution containing 1mM DCA, TUNEL positive cells were seen mainly in the more superficial layers of the epithelium but to a lesser extent in cells from the prickle cell layer. In contrast to the rabbit epithelium, TUNEL positive cells were remarkably observed in the proliferative basal layer of the human epithelium (figure 7 F). In this condition apoptotic cells accounted for a 52.4±4.1 % of the total epithelium. After 120 minutes, the weakly acidic solution at pH 5.0 did not induce apoptosis (figure 7G) and only 1 % of epithelial cells were apoptotic.
DISCUSSION

In the present study we tested the hypothesis that acidic and weakly acidic solutions containing the bile acid DCA can impair the esophageal mucosal integrity in vivo provoking long lasting effects of several hours and even days. For that, we performed functional, histological and ultrastructural measurements. Since this hypothesis cannot be performed in healthy volunteers due to the complexity and ethical issues, we used an esophageal perfusion model in anesthetized rabbits that we have established and described before.(6)

We showed that: (1) esophageal epithelial integrity is highly impaired after perfusion of an acidic solution at pH 1.0 and a weakly acid solution at pH 5.0 containing 500 µM of the bile acid DCA; (2) an impaired esophageal epithelium is still present after 24 hours of perfusion and only recovered after 48 hours in the case of the acidic solution; (3) the solution containing DCA but not the acidic solution induces apoptosis of the epithelial cells; (4) apoptosis is also induced in human esophageal epithelium after in vitro exposure of DCA.

These results may indicate that the presence of impaired esophageal integrity in GERD can be caused by a rapid and acute effect of continuous reflux events but also by the mucosal contact of harmful luminal agents that occurred hours or even days before.

Several studies suggest that the presence of an impaired epithelial integrity is a key contributor in the pathogenesis of GERD not only in patients with erosive esophagitis but also and more interestingly in patients with NERD. An impaired esophageal epithelial integrity has been found in non-eroded areas of erosive esophagitis patients (18) but more controversial is the presence of such alteration in patients with NERD.(36; 39; 41; 42) The
current hypothesis on how acid is perceived in NERD patients, and also applicable to subjects
with erosive esophagitis, is that protons present in the acidic refluxate cross the esophageal
epithelium due to the presence of an impaired integrity. Then, protons can reach sensory
nerve endings present in deeper layer of the epithelium and/or lamina propria.(1) Based on
in vitro experiments and scarce in vivo animal and human research evaluating the short term
effect of perfused solutions, we know that different luminal factors can impair epithelial
integrity with or without provoking visible erosions.(6; 25; 28) Perhaps, based on these
studies, the general believe is that the continuous effect of the gastric content in the
esophagus causes a rapid and caustic effect on the esophageal epithelium. Nevertheless, the
possibility that harmful events can provoke long lasting alterations in the epithelial integrity
is also plausible and has never been explored before.

As reported in rabbits and more recently also in humans, esophageal perfusion with a very
acidic solution (pH 1.0-1.6) can impair esophageal epithelial integrity.(6; 8; 28) In the present
study we confirmed this finding in our rabbit model of esophageal perfusion but also we
found that a low concentration of the bile acid DCA (200 µM) impairs epithelial integrity,
reaching a similar magnitude as the acidic solution at 500 µM concentration. To the best of
our knowledge, this is the first evidence showing a functional effect in vivo of this bile acid
on epithelial integrity.(9; 32) Although studies obtaining esophageal aspirates showed
increased DCA in patients with GORD off PPIs,(26) in our knowledge the gastric
concentration of DCA was never reported. Studies of continuous 24h aspiration of gastric
juice show that total bile acid concentration has a circadian variation. In healthy volunteers,
the concentration can rise from 1 mM in fasting conditions to 3-4 mM after a meal. (10) Bile
acids are conjugated with taurine and glycine and the presence of unconjugated forms in
volunteers represents the minority, but the presence of conjugated DCA represents around
In patients taking PPI, the esophageal mucosa is exposed to weakly acidic reflux and some of the patients present gastric bacterial overgrowth, which results in an increased proportion of unconjugated bile acids from 20 to 80\%. Then, it is quite likely that in patients on PPI, an important concentration of DCA is present in the gastric juice.

More interesting is that in both, acidic and weakly acidic conditions containing DCA, no microscopic or histologic alterations could be observed. Nevertheless, at the ultrastructural level we clearly observed the presence of DIS at the basal cell layer of the epithelium in both conditions. In contrast, DIS were also found in the prickle cell layer of the epithelium only after perfusion with the acidic solution. Although in humans DIS are mainly assessed in the basal (also called proliferative) cell layer, recent studies show that some GERD patients have widening of the intercellular spaces also at more superficial layers, indicating that acid can be the agent or one of the agents responsible for this alteration. More striking is that a solution at pH 5.0 was able to induce DIS only in the basal cell layer of the epithelium without changes in TEER indicating that in some conditions, ultrastructural changes do not translate to functional alterations as we already reported before in vitro in rabbit tissue.

Despite the similar functional acute effect of the acidic and weakly acidic solution on TEER and PD, the long-term effect was markedly different. An impaired epithelial integrity is still present after 24 hours of the esophageal perfusion with the acidic and weakly acidic solution. Nevertheless, after 48 hours of the esophageal perfusion the epithelial integrity is recovered when the acidic solution was perfused but remains still slightly impaired after the perfusion with the weakly acidic solution. These data clearly show that the impaired epithelial integrity observed in patients with GERD is not provoked only by the direct caustic effect but likely also due to a long lasting effect of harmful reflux events. The discrepancy...
observed in the recovery of both solutions can be explained by the fact that the weakly acidic solution containing DCA provokes apoptosis as observed by TEM and further confirmed by TUNEL staining. These cells have to be replaced by the cells of the proliferative cell layer to renew this part of the epithelium. Moreover, the proliferative basal cell layer that is in charge of keeping the homeostasis of the epithelium is prominently disturbed 24 hours after the perfusion with the weakly acidic solution as had been shown by TEM studies. Then, the stem cell niche, recently characterized in the esophageal epithelium, (16) has to regenerate the proliferative cell layer and to restore the epithelium as had been seen at 48 hours after perfusion. In contrast, the alterations induced by the acid solution are not compromising the viability of the epithelial cells, are reversible and are caused by the osmotic force for water movement into the intercellular space. (37) Although this finding was never described in the esophagus, in colonic epithelial cells camptothecin can cause a decrease of TEER and an increase in fluxes of the paracellular marker mannitol and increase the number of apoptotic cells. (3; 4) The present study is the first to link the presence of apoptosis with an impaired epithelial barrier function in the esophagus.

The poor correlation observed between esophageal epithelial integrity and acid exposure time (19; 29) may indicate that other luminal agents perhaps inducing apoptosis can be relevant in GERD pathophysiology. This premise can be supported by the increased number of apoptotic cells in the superficial cell layer of the epithelium of patients with GERD and their reduction following anti-reflux surgery. (40) To our knowledge, the direct effect of DCA in vivo causing apoptosis and provoking long lasting alterations was never reported before. Only few reports in vitro in epithelial cell cultures described this phenomenon. (13; 43) Our in vitro results show that the induction of apoptosis by DCA is preceded by the impaired
epithelial integrity indicating that these two phenomena are separated over time. Nevertheless, our results suggest that the presence of apoptotic cells can contribute to the long lasting and incomplete recovery of the epithelial integrity. The exact mechanism of DCA-induced apoptosis in normal human epithelial cells is unknown, but activation of the Na+/H+ exchanger and pathways involving mitochondrial function may be involved. The capability of bile acids to induce apoptosis is not restricted to DCA as has been shown by Zhang et al., indicating that the presence of apoptotic cells in the epithelium may be a marker of DGER. Although the direct extrapolation of the present findings from our rabbit model to GERD patients has to be done with caution, our findings suggest that DCA is able to impair esophageal integrity and to induce apoptosis in humans also, since we could reproduce the same results using human esophageal epithelium.

Finally, we recognize that this study has its limitations. An ideal model looking at effects of chronic and intermittent esophageal exposure to different solutions or modified gastric juice should approach the real life situation of GERD patients. However, several separate exposures during the course of the day, with sequences of acidic, weakly acidic, bile or mixed reflux episodes, for several consecutive days or months are not possible to obtain in any model. Therefore we chose to reproduce certain aspects of refluxate exposure that can be found in refractory patients taking PPI. Simultaneous pH and Bilitec measurements show that GERD patients not responding to PPI have a normal esophageal acid exposure time (most of the reflux events with a pH higher than 4 units) but pathological duodenal reflux exposure of around 14% independently of the presence or absence of erosions in the esophagus. In other words, during 3 h a day the esophagus of these patients is exposed to bile acids in a weakly acid environment. DGER is considered to be pathologic when the % of the exposure time is higher than 4.6, that is the equivalent to 1 h a day.
Based on these and others findings mentioned above, we decided to perfuse the esophagus during 30 minutes with a weakly acidic solution containing the bile acid DCA.

In conclusion, our findings indicate, for the first time, that the presence of an impaired esophageal epithelial integrity present in GERD patients may be caused by a long lasting effect and/or to an incomplete repair mechanism induced by luminal agents. Despite the similar potentially damaging effect, acidic and weakly acidic solutions containing DCA impair esophageal epithelial integrity by two different mechanisms. The induction of apoptosis by DCA can explain the slow and incomplete recovery of the epithelial integrity. Further investigations to reveal the mechanism involved in DCA-induced impaired integrity and apoptosis of the epithelial cells may therefore be important for the development of therapeutic strategies. Our findings challenge the classical paradigm that the presence of altered esophageal mucosal integrity is solely explained by a direct caustic effect of luminal agents. Impairment of the epithelium can also be a sign of a prolonged and sustained reaction induced by earlier exposure of luminal agents.(33)
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Competing interests None.

Ethics approval The procedure for the animal experiments was approved by the ethics committee for animal experiments of the Catholic University of Leuven, Belgium. The part of the study involving human tissue from organ donors was approved by the Ethical Committee of the Catholic University Leuven. Belgian legal aspects concerning the research on tissue from a death organ donor were followed and ethical approval was granted by the local ethical committee (University of Leuven; S56978-ML10868).


Figure legends

Figure 1. Acute effect in vivo of an acidic solution at pH 1.0 and a weakly acidic solution at pH 5.0 containing DCA on TEER and PD. A and C: effect of the solutions at pH 1.0 and pH 5.0 containing DCA 500 µm. B and D: effect of the solutions at pH 5.0 containing DCA 200 µm and DCA 500 µm. Transepithelial electrical resistance (TEER), potential difference (PD), deoxycholic acid (DCA). * p<0.05 vs. pH 7.2, ** p<0.01 vs. pH 7.2, # p<0.01 vs. pH 5.0.

Figure 2. Correlation between TEER and PD after in vivo esophageal perfusion with solutions at pH 7.2, 5.0 (with and without DCA) and pH 1.0. Transepithelial electrical resistance (TEER), potential difference (PD).

Figure 3. Representative TEM photographs of the basal (A-D) and prickle cell layer (E-H) of the esophageal mucosa of rabbits perfused with pH 7.2 (A, E), pH 5.0 (B, F), pH 1.0 (C, G) and DCA 500 µM (D, H). The acidic solution at pH 1.0 provokes DIS in the basal (C) and prickle cell layer (G) of the epithelium. The weakly acidic solution at pH 5.0 induces DIS in the basal cell layer of the epithelium (B, F). DCA provokes DIS in the basal (D) and induces apoptosis in the prickle cell layer (H). Transmission electron microscopy (TEM), deoxycholic acid (DCA).

Figure 4. Representative photographs of TUNEL staining (A, B) and TEM (C, D). TUNEL staining confirmed the presence of apoptotic cells in the prickle and more superficial cell layers of the esophageal epithelium in animals perfused with deoxycholic acid 500 µM (A,C) compared with the acidic pH 1.0 solution (B,D). Apoptotic cells are not present in the prickle cell layer of the mucosa exposed to pH 1.0.

Figure 5. Long term effect in vivo of 30 minutes esophageal perfusion of an acidic solution at pH 1.0 and a weakly acidic solution at pH 5.0 containing DCA on TEER and PD. A and C: effect
of the solution at pH 1.0. B and D: effect of the solutions at pH 5.0 containing DCA 500 µm.

Deoxycholic acid (DCA), transepithelial electrical resistance (TEER), potential difference (PD).

* p<0.05, ** p<0.01, *** p<0.001.

**Figure 6.** Representative transmission electron microscopy photographs of the basal cell layer of the esophageal mucosa of rabbits after perfusion with pH 1.0 (top photographs) and DCA 500 µM (bottom photographs) at t=0 (A, D) and after 24 hours (B, E) and 48 hours (C, F) recovery. Deoxycholic acid (DCA).

**Figure 7.** Effect *in vitro* of the weakly acid solution containing DCA on TEER and apoptosis in rabbit and human tissue. A) % of change in TEER. Representative photographs of TUNEL staining after the exposure of rabbit B) and C) human oesophageal epithelium to the weakly acid solution containing DCA. Deoxycholic acid (DCA), transepithelial electrical resistance (TEER).