Acute-phase response in pigs undergoing laparoscopic, transgastric or transcolonic notes peritoneoscopy with us or eus exploration

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Abstract

Background : **Laparoscopic surgery is associated with reduced surgical trauma, therefore with acute-phase response of lower magnitude as compared with open surgery. We hypothesized that NOTES might induce reduced immune response as compared with laparoscopy.**

Objective : **To compare acute-phase reactants in a controlled trial of laparoscopic peritoneoscopy and ultrasonography versus transgastric or transcolonic NOTES peritoneoscopy and intraperitoneal endoscopic US.**

Methods : **Eighteen pigs were divided in 3 groups : laparoscopy, transgastric and transcolonic NOTES. Serum levels of IL-6 and TNF-α were determined preoperatively and at day 2. Serum levels of haptoglobin and IL-6 mRNA levels from isolated white blood cells were measured by RT-PCR at days 0, 1, 2 and 7. Necropsy was performed at sacrifice, with peritoneal fluid microbiological analysis, macroscopic and microscopic examinations on gastrotomy/colotomy or abdominal wall closure sites, liver and parietal peritoneum biopsy sites and any area suggestive of infection.**

Results : **The groups were similar with regards to peritoneoscopy completeness, ultrasonographic examination and biopsies. The duration of NOTES procedures was significantly longer than laparoscopic procedures. Minor complications were observed in most animals by macroscopic and microscopic examination, but NOTES procedures were associated with severe complications in 3 pigs (fistula, abscess, mortality). No significant differences in acute-phase reactants levels were found between groups.**

Conclusions : **No significant difference in the acute-phase reactants could be demonstrated between surgical and NOTES procedures. NOTES was however associated with more severe septic complications. Optimal closure remains a challenge and better devices are needed to avoid them.** (Acta gastroenterol. belg., **2012**, 75, **28-34**).

Key words : NOTES, Natural Orifice Translumenal Endoscopic Surgery, immune response, peritneoscopy, endoscopic ultrasonography, laparoscopy.

Introduction

Surgical trauma causes significant alterations in host immune function. Compared with open surgery, laparoscopic surgery is associated with reduced postoperative pain and more rapid return to normal activity. The first NOTES (Natural Orifice Transluminal Endoscopic Surgery) study was published in 2004 by Kalloo *et al*. (1), describing transgastric peritoneoscopy in a porcine model. The first transcolonic NOTES approach was reported by Pai *et al*. in 2006 performing cholecystectomy in pigs (2). A potential advantage of NOTES results from the further reduction of surgical trauma when compared with laparoscopic and open surgery, which means that NOTES should result in even less adhesion formation (3), a lower stress response, lower pain scores (4), and an enhanced postoperative recovery. Few studies have compared the potential benefits of NOTES approaches to laparoscopy (5-7), and no definite conclusions could be drawn about the superiority of either method. Theoretically, if NOTES induces fewer traumas than laparoscopy, a lower post-procedural inflammatory response should be expected. The cytokines Tumour Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6), are known to be major mediators of the acute-phase response to inflammation. TNF-α contributes to the acute-phase response, but is primarily responsible for non-hepatic manifestations which include fever and tachycardia (8). Serum IL-6 levels are early and sensitive markers of tissue damage. They rise in proportion to the surgical trauma, associated injury, and development of major complications, blood loss, and length of operation. Haptoglobin is an acute-phase protein, synthesized mainly in the liver, providing antioxidant and antimicrobial activity. Changes in serum concentration can occur in several pathological conditions : increases in inflammation, infection and malignancy, decreases in haemolytic conditions (9,10).

The first application for NOTES relates to peritoneoscopy that may become an alternative to laparoscopy (1). The transluminal approach could be particularly important for morbidly obese patients and others at high risk of complications from open and laparoscopic surgery. In some groups of patients, such as patients with periampullary and liver malignancies,

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laparoscopic US has been shown to have additional benefit over laparoscopy alone and increases the yield of laparoscopy (11-15). If NOTES peritoneoscopy is to become an alternative to diagnostic laparoscopy, it must be as accurate as laparoscopy and comparable from a diagnostic standpoint, including intraperitoneal EUS.

The aim of the study was to compare the systemic production of cytokines and acute phase reactants, and the feasibility to combine US to peritoneoscopy, in a controlled trial of transgastric (TG) and transcolonic (TC) endoscopic peritoneoscopy combined with intraperitoneal EUS versus laparoscopic (LAP) peritoneoscopy with US in a survival porcine model.

Material and methods

The study was conducted in the research laboratory of experimental surgery of the Université Catholique of Louvain (UCL), with the approval of the UCL Ethics Committee for Animal Care (réf. : UCL/MD/2008/011). All interventions were performed under presumed aseptic conditions, general anaesthesia with the pig in supine position, intubated and mechanically ventilated.

Animals and preoperative preparation

Female Landrace pigs (n = 18) provided by Rattlerow Seghers were used in the study. All pigs were housed and cared at the laboratory of experimental surgery (UCL), according to the guidelines of the Belgian Ministry of Agriculture and Animal Care.

Animals were fasted for 24 hours before intervention, with free access to water. Anesthesia of Landrace pigs was induced intramuscularly by Zoletil® 100 (Virbac sa, Carros, France) at a dose of 6 mg/kg and Rompun® (Bayer AG, Leverkusen, Germany) at a dose of 2 mg/kg. Placement of an ear intravenous catheter was performed, and after intubation, inhalation anaesthesia was maintained by Isoflurane (Isoba(r) Vet, Intervet/Schering-Plough Animal Health, Belgium), nitrous oxide and oxygen. An intramuscular antibiotic prophylaxis (Cefazolin 1 g) was given perioperatively. Pigs were fed immediately after surgery and were sacrificed one week later with injection of T61® (Intervet/Schering-Plough Animal Health, Belgium).

Procedure and technique

NOTES and laparoscopic procedures were carried out in 18 female pigs, divided in 3 equal groups assigned to either a classic diagnostic laparoscopy, or a transgastric or transcolonic NOTES abdominal exploration.

Laparoscopy

Procedures were performed by two surgeons (C.R., B.N.) experienced in laparoscopic techniques. Pneumoperitoneum was obtained by using a Verres needle with CO2 insufflation until 12 mmHg. A 10-mm trocar was introduced in the midline. After general inspection of the abdominal cavity, 2 additional trocars were inserted under direct visual control : one 5-mm in the right flank and one 10-mm in the left flank. A 3 incisions approach was chosen to ensure a more complete approach for peritoneal examination, as it is routinely done in our institution for peritoneal cancer staging. This approach ensures a better view of the cavity and grasping of organs, and easier hemostasis in case of bleeding after liver or peritoneal biopsies.

A meticulous exploration of the following 9 areas of the abdomen was carried out : right and left hypochondrium, epigastric area, right and left flank, mesogastric area, right and left iliac fossa and hypogastric area. Laparoscopic ultrasonography (LUS) of the liver was performed with a rigid 10-mm LUS probe introduced through the median port. A macroscopic peritoneal biopsy was taken at the level of the anterior abdominal wall as well as a liver biopsy with a biopsy grasper. Finally, after instillation of 20 cc of serum saline in the right inferior quadrant, the fluid was reaspirated for bacterial count.

Operating time was calculated from the incision to the closure of the skin.

NOTES

All endoscopic procedures were carried out by two experienced endoscopists (P.H.D., C.P.R.Y.). Endoscopes and accessories were prepared with high-level disinfection (Cidex OPA ; Ethicon, Inc, Irvine, California, USA), followed by gas sterilization with ethylene oxide. Pneumoperitoneum was obtained by using a Verres needle with CO2 insufflation until 12 mmHg.

A standard upper endoscope (GIF-160, Olympus Belgium, Aartselaar, Belgium) was advanced into the pig's stomach or colon. Gastric or colonic cleaning was performed with water and chlorexidine. The endoscope was positioned adequately, and the anterior wall of either the stomach or the colon was located by transillumination. An endoscopic needle knife (KD-10Q-1.A ; Olympus Belgium, Aartselaar, Belgium) was used to create an initial 2-mm incision in the anterior wall of the hollow viscus, with pure-cut current. This opening was enlarged by a 15-18-mm balloon (CRE, Boston Scientific Corp, Galway, Ireland) and the endoscope was introduced into the peritoneal cavity.

Meticulous exploration of the 9 abdominal areas previously described was carried out, and macroscopic peritoneal biopsy was performed at the level of the anterior abdominal wall as well as a liver biopsy with a endoscopic biopsy forceps. Endoscopic ultrasonography of the liver was performed with a 12 Mhz miniprobe introduced through the endoscope (Fujinon, ONIS, Belgium). Finally, after instillation of 20 cc of serum saline in the right inferior quadrant, the fluid was aspirated for bacterial count.

Closure of the gastrotomy or colotomy site was performed by use of T-bar anchors (Olympus, Tokyo, Japan) and clipping in case of persistent digestive wall leakage (EZ-clips, Olympus Belgium, Aartselaar, Belgium).

Operating time was calculated from the initial insertion to the final withdrawal of the endoscope from the mouth of the pig.

Clinical assessment : pigs were followed clinically by measure of weight at days 0 and 7, and daily observation of feeding, tonicity and general behaviour.

Laboratory analyses

Serum levels of IL-6 and TNF-α were measured by commercially available ELISA kits (Porcine IL-6 Quantikine ELISA kit, Porcine TNF-alpha/TNFSF1A Quantikine ELISA kit, R&D systems, Minneapolis, MN, USA,) at days 0 (preoperative) and 2, following the manufacturer's instructions. Haptoglobin levels were determined from pig serum samples by spectrophotometry (16).

Bacteriologic sampling in the peritoneal fluid was performed at days 0 and 7, and sterility checked in the Microbiology Department.

Lymphocytes isolation

All manipulations were done at room temperature and all centrifugation steps at 20°C. Tubes containing EDTA blood were diluted twice in Hanks' balanced salt solution (HBSS) (Invitrogen, Life technologies S.A. Merelbeke, Belgium). Diluted blood was carefully layered upon a FicollIsodium metrizoate gradient (Ficoll PaqueTM, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and lymphocytes were isolated following the manufacturer's instructions.

RNA isolation and quantitative polymerase chain reaction (PCR)

To increase the sensitivity of evaluation of inflammatory response, quantitative PCR (on circulating white blood cells) was used to determine the concentrations of IL-6 RNA at days 0 (preoperative), 1, 2 and 7. Total RNA was prepared from lymphocytes using TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Quantitative PCR analysis was performed with the GeneAmp® 5700 Sequence Detection System andsoftware (Applied Biosystems, Den Ijssel, Netherlands) using Cybergreen fluorogenic probes. GAPDH mRNA was chosen as an internal standard. All primers were designed using the Primer Express™ design software (Applied Biosystems). The following primers were used :

IL-6 sense : GACAAAGCCACCACCCCTAA ; antisense : CTCGTTCTGTGACTGCAGCTTATC. GAPDH sense : CAGCAATGCCTCCTGTACCA ; antisense : GCCGAAGTTGTCATGGATGA.

PCR reactions were performed according to the standardized thermal profile of the system previously set by the manufacturer. All tissue samples were run in duplicate at the same time in a single 96-well reaction plate (MicroAmp® Optical, Applied Biosystems) using appropriate primers and probes. Quantification was obtained according to the ΔΔCT method as specified by the manufacturer. The final result of each sample was normalized to its respective GAPDH value (17).

Necropsy

Necropsy was performed at day 7 after the intervention. The peritoneal cavity was examined for injury to adjacent organs, signs of infection, or adhesions. The sites of gastric/colonic and abdominal wall closure were examined for any residual defect. Macroscopic and microscopic examinations of specimens obtained from the gastrotomy/colotomy sites, abdominal wall closure sites, liver and parietal peritoneum biopsy sites and any area suggestive of infection were performed.

Pathological evaluation

Specimens were fixed in 10% formalin and paraffin embedded to prepare slides. Slides previously stained with hematoxylin and eosin (HE) were randomly reviewed by an experienced pathologist (A.J-M.). Histological evaluation was based on acute inflammation (neutrophilic infiltration), with or without abscess formation (necrosis and dense infiltrate of neutrophils), chronic inflammation (lymphoplasmocytic infiltrate), foreign body granulomas, healing and scarring (active collagen deposition, neovascularisation, fibroblastic proliferation).

Statistical analyses

Results are expressed as mean and standard error of the mean (SEM).

Each animal was studied by several samplings, and the results were thus correlated for the same animal : so linear regressions were studied by generalized estimating equation (GEE) by quasi-least squares (QLS). We selected a model with a common correlation for all the measures, and the correlations were computed according to Chaganty and Shults (18). We used the sandwich variance matrix augmented by the correction proposed by Morel, Bokossa and Neerchal (19) which is convenient for small samples. Significance was accepted at the $p < 0,05$.

Results

Eighteen female pigs with a mean weight of 29,5 ± 2,1 kg were included in this trial (6 in the laparoscopy, 6 in the transgastric NOTES, 6 in the transcolonic NOTES group).

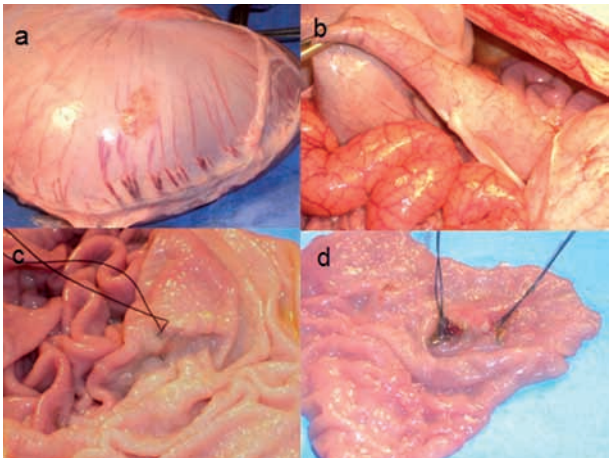

Fig. 1. — Closure with T-bars. Closure of the stomach perforation viewed from outside (a), from inside (c). Closure of the colon perforation viewed from outside (b), from inside (d).

Assessment of endoscopic and surgical procedures

LAP and NOTES peritoneal access, US examination of all quadrants of abdomen, and peritoneal and liver biopsy were performed in all pigs. During TG procedure, visualisation of the nine abdominal

areas was feasible but liver was difficult to access in one pig. During one of the TC procedures, exploration of the abdominal cavity was technically difficult because of excessive bowel insufflation.

The gastric and colonic incisions were easily performed without complications, as well as laparoscopic approaches. Mean operating time for TG NOTES, TC NOTES and laparoscopic surgery was 62, 91 and 24 minutes, respectively ($p < 0,05$ between LAP and TC or TG NOTES).

Closure with T-bars and clips was successful in all pigs (Fig. 1). Two to five T-bars were used per closure and no complications were seen during the NOTES procedure. When clips were needed, 5 to 8 clips were placed, except in one TC pig in which more than 10 clips were applied because of poor visibility.

Clinical evaluation

Nine pigs in the NOTES TG and TC groups gained weight (0,7 to 4,35 kg), and three lost from 0,7 to 1,8 kg. In the LAP group, three pigs gained weight (0,45 to 2,05 kg), whilst three pigs lost from 0,25 to 1 kg. Observation of feeding, tonicity and general behaviour was considered uneventful, except in one TC NOTES pig which developed peritonitis and died 72 hours later.

Pathology

Necropsy results at day 7 are summarized in Table 1. In the TG group, one pig had a deep ulcer of 8 mm covered with omentum, and another one developed a colo-gastric fistula, caused by inappropriate T-bar placement. Minor adhesions were demonstrated in 3 pigs in the NOTES groups and in the TC NOTES group, one pig had an abscess and adhesions in mesentery. In LAP group, 3 pigs developed small parietal and/or liver biopsy site collections. Two pigs developed adhesions related to the medial parietal scar and between small bowel loops, and one had a colonic wound in regard to the medial scar. Microscopic findings at day 7 are shown in Table 2. They confirmed the macroscopic lesions and showed more infected sites, ulcers and vascular thrombosis in all groups studied. In the LAP group, lesions were only noted at the parietal incision sites (necrosis, microabscesses, inflammation, microscopic fistula).

Laboratory

Haptoglobin (Fig. 2) levels were measured at days 0, 1, 2 and 7 and showed a significant increase on day 1 and 2 compared to day 0 ($p = 0,01$) in all groups, but no significant difference between groups. A more important rise of haptoglobin was observed in the pigs with minor and severe complications (TC3 which died of peritonitis,

Table 1. — **Results of necropsy**

	LAP	TG NOTES	TC NOTES
Minor complications			
Abdominal wall collection	1		
Liver biopsy site collection	3		
Adhesions	1	2	2
Severe complications			
Intra-abdominal abscess			1
Deep ulcer		1	
Gastro-colic fistula		1	
Death			1

Number of pigs which presented the necropsy findings referred in the first column. LAP indicates laparoscopic intervention. TG and TC indicate Transgastric and Transcolonic NOTES procedures, respectively.

Table 2. — **Results of histopathology**

	LAP	TG NOTES	TC NOTES
Intra-parietal abscess	4	2	2
Intra-abdominal abscess		1	2
Ulcer		2	1
Microvascular thombosis			1

Number of pigs which presented the histopathological findinsg referred in the first column. LAP indicates laparoscopic intervention. TG and TC indicate Transgastric and Transcolonic NOTES procedures, respectively.

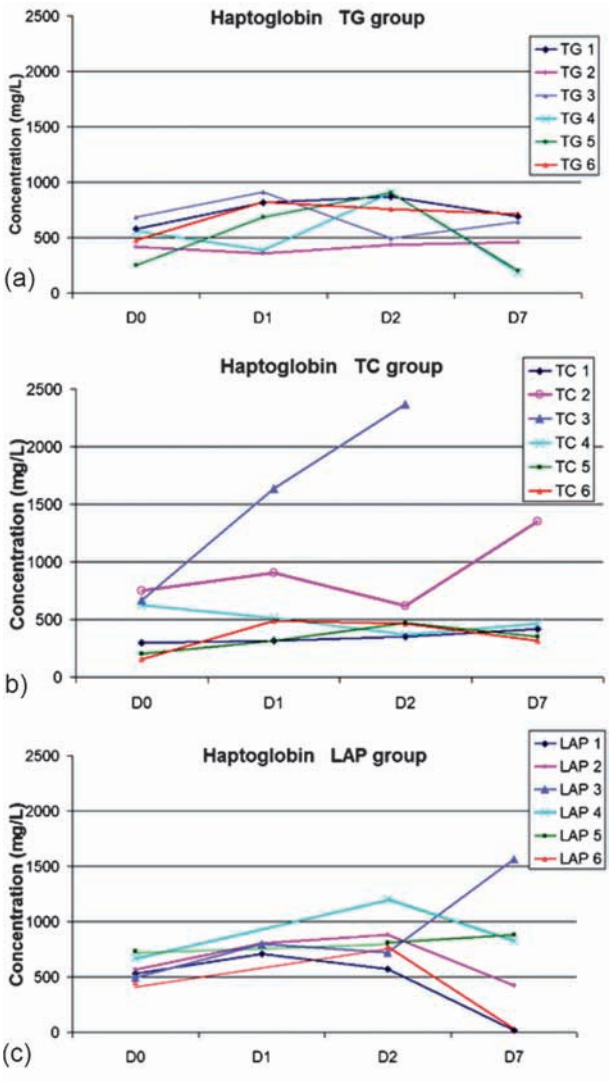

Fig. 2. — Evolution of haptoglobin serum levels from day 0 to day 7 in each group. (a)Transgastric (TG), (b) transcolonic (TC), (c) laparoscopic (LAP) groups.

TC2 with an abscess and adhesions in mesentery, LAP3 and 4 with macroscopic small collection and microscopic liver and parietal abscesses, respectively).

No IL-6 serum levels could be detected by ELISA. In order to increase sensitivity, IL-6 mRNA was measured by quantitative PCR and results are shown in Figure 3. No significant differences were seen between days and groups. Values at days 0 and 2 were respectively 0,66 ± 0.43 and 1,35 ± 1,15 IL6/GAPDH for TG group, 0,80 ± 0,62 and 0,72 ± 1,07 IL6/GAPDH for TC group ; 0,75 ± 0,30 and 0,91 ± 0,74 IL6/GAPDH for LAP group. Similar values were observed on day 1 and 7 without any significant differences.

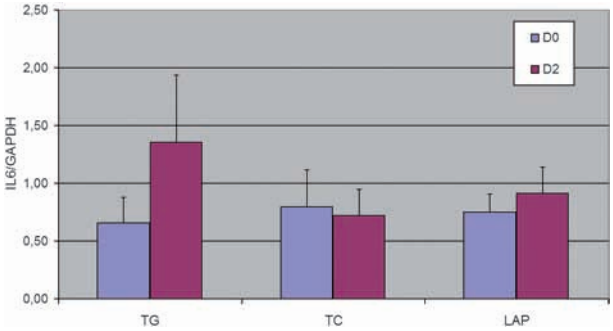

Fig. 3. — Results of IL-6 RNA levels, extracted from circulating white blood cells, obtained by PCR. Comparison between transgastric (TG), transcolonic (TC) and laparoscopic groups (LAP).

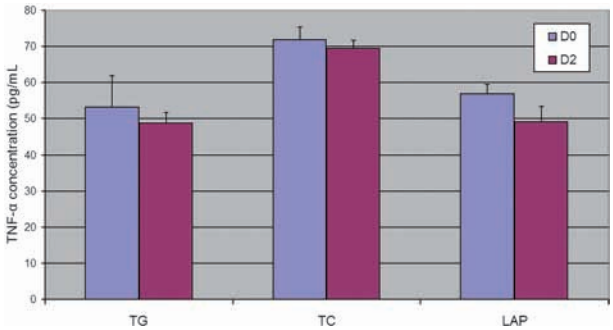

Fig. 4. — Results of TNF-alpha serum concentrations obtained by ELISA. Comparison between transgastric (TG), transcolonic (TC) and laparoscopic groups (LAP) at days 0 and 2.

Concentrations of TNF-alpha obtained by ELISA (Fig. 4) showed no significant differences between days 0 and 2 with respective values of 53,28 ± 9,87 and 48,82 ± 9,87 pg/mL for TG group, 71,85 ± 17,42 and 69,56 ± 23,16 pg/mL for TC group, 56,96 ± 13,25 and 49,20 ± 7,09 pg/mL for LAP group.

Bacteriologic samplings in the peritoneal fluid, at day 0 at the end of the peritoneoscopy and after having taken the parietal and liver biopsies, and at day 7 at sacrifice,

were negative in all pigs except in two pigs : in the TC2 which developed the abscess (*E. Coli*) and in the LAP6 with the colic wound (*Streptococcus viridans*, coagulase-negative Staphylococcus).

Discussion

The main objective of our study was to compare the acute-phase response in transgastric and transcolonic NOTES vs. laparoscopic peritoneoscopy including ultrasonography or endosonography. We measured three major mediators of the acute-phase response including haptoglobin levels, serum TNF-α, and IL-6 concentrations (measured by ELISA), as well as IL-6 mRNA levels obtained from isolated circulating lymphocytes. No significant differences could be observed between the different groups of pigs included in the study. Levels were low for most of the mediators at all examined time points. Haptoglobin was shown to be the most sensitive marker predicting septic complications in four pigs and should therefore be considered as the most reliable dosage to be performed in pig surgical or endoscopic experiments. Recent reports comparing cytokine levels between laparoscopic and NOTES groups could not find any significant difference during peritoneoscopy, even during viscerectomy (5,7,20). This might be explained by low or undetectable levels of cytokines in particular serum IL-6 concentration when measured by ELISA method. Therefore we measured, by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), IL-6 mRNA levels in circulating lymphocytes to increase the sensitivity of evaluation of inflammatory response. By doing so we were able to detect differences between days and groups but without reaching a statistical significant difference. This might be due to the variability observed in the measures and the limited number of pigs included in our trial. Combination of peritoneoscopy, with parietal and liver biopsy and ultrasonography may also have induced lesser trauma of stress than a surgical procedure such as a cholecystectomy. Combination of EUS and NOTES was advocated to guide access and therapy (21), but also to provide a complete staging of the peritoneal cavity and liver comparable to laparoscopy plus US in its diagnostic accuracy. Voermans *et al*., when evaluating the feasibility of NOTES EUS, showed that intraperitoneal ultrasonography induced a longer procedural time (22). Hazey *et al*. reported that NOTES peritoneoscopy took about twice as long as diagnostic laparoscopy in humans, even without intraperitoneal US (23). A longer period of stress due to a prolonged procedure was previously shown to cause a higher stress response (24). The duration of our NOTES procedures, all performed by experienced endoscopists, was also significantly longer than the laparoscopic approach. In our study, the prolonged duration was not due specifically to peritoneal access (44%), US examination or closure time (35%), but rather to the combination of these manipulations. The prolonged procedural duration in the NOTES groups had however no influence on inflammatory response.

The second objective of our study was to evaluate the feasibility and completeness of NOTES peritoneoscopy with endoscopic ultrasonography as compared with the standard laparoscopic ultrasonography approaches, including efficiency and safety of wall closure. The 3 groups were similar with regards to peritoneoscopy completeness, ultrasonographic examination and liver and peritoneal biopsy. Although the probes used during NOTES (12 MHz) and laparoscopy (7.5 MHz) had different depth of penetration, both were able to analyse the liver surface and parenchyma. Our study was however not aimed at comparing different types of US probes. In most pigs, closure was effective with minimum adhesions and no infection controlled by microbiological sampling of the peritoneal fluid, macroscopic examination of the peritoneal cavity. Due to early necropsy (at 7 days rather than 14 days as reported in other papers (3,7,25,29)), microscopic examination showed microabscesses and ulcers, in the parietal, gastric, and colonic access and closure sites in 82% and 62% of NOTES and laparoscopic procedures, respectively. These minor complications or observations were not reflected in by an increased inflammatory stress response. We also observed major complications such as a gastro-colic fistula in a TG NOTES, an abscess in a TC NOTES, and a peritonitis resulting in death in another TC NOTES. These complications were due to ineffective closure in the colon and inappropriate placement of T-bars. These potential concerns were raised by several studies investigating various closure devices, especially with full-thickness needle-puncture technique associated with a risk of injury to adjacent viscera and vessels (26-28). Safe and reliable devices and methods for closure are essential, especially when dealing with inadequately sterilized contents (29). Indeed antibacterial lavage, prophylactic injection of antibiotics and high level instrument disinfection, may not be sufficient in coprophagic pigs with rudimentary gastric and colonic cleaning, and immediate refeeding after intervention. Use of clips and T-bars in our experiments might not have provided an optimal closure, as was shown by Voermans *et al*. (22) who experienced inadequate leak testing when using clips or T-bar closure.

Limitations in our study are the limited sample size inducing an alpha error to detect significant change in acute-phase reactants initiated by surgical trauma and the related complications, and the fact that results analysis was done on an intention-to-treat basis therefore including pigs with major complications, which might have increased the variability of cytokine and haptoglobin values.

In conclusion, no significant difference in the acute-phase reactants could be demonstrated between surgical and NOTES peritoneoscopy with ultrasonography or endosonography. The duration of transgastric and transcolonic NOTES procedures was significantly longer, but

without affecting cytokines or haptoglobin levels. More severe complications, including mortality and abscesses, were observed in the NOTES pigs, further emphasizing the importance of improving the safety of wall closure. Better devices than clips and T-bars are needed to avoid the complications observed in our study.

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