Szent István University

Postgraduate School of Veterinary Science

The role and options of gastrointestinal endoscopy in the gastroenterological diagnostics and therapeutics in dogs

Theses of PhD dissertation

Roland Psáder DVM

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Szent István University

Postgraduate School of Veterinary Science

Supervisor:

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Dr. Ágnes Sterczer PhD

associate professor

Szent István University, Faculty of Veterinary Science

Department and Clinic of Internal Medicine

……...

Roland Psáder DVM

1. Introduction and aims of the studies

Gastrointestinal endoscopy as a complementary diagnostic method plays an important role in the early detection of a variety of gastrointestinal disorders. In many cases it is the most effective and minimally invasive procedure that allows detection of diseases macroscopically and in the subclinical stage. Gastroduodenoscopy is excellent for follow-up the patient's recovery and for investigation the nasojejunal feeding tube's applicability.

I would like to demonstrate the role and possibilities of gastrointestinal endoscopy in the gastroenterological diagnostics and therapeutics in dogs.

According to the human literature the application of jejunal feeding tube inserted into the second jejunal loop, behind the Treitz ligamentum the pancreas stimulation could be avoided omitting the cephalic and gastric phases putting the pancreas into the rest (Bodoky et al., 1991; Harsányi et al., 1991; Oláh et al., 2002). The gallbladder motility is controlled by neuro-hormonal mechanisms, in the same way as pancreas secretion. Sterczer et al. (1996) published the ultrasound-guided method for measuring the gallbladder contraction. This method can be useful for follow-up the post-feeding gallbladder contraction referring the pancreas function. Endoscopically guided nasojejunal tube placement in dogs was described by our team earlier (Pápa et al., 2009). In the first part of the thesis the effect of enteral feeding on gallbladder function was investigated in dogs. The objective of the study was to compare the effect of a cholagogue meal applied into four anatomical regions (jejunum, duodenojejunal junction, descending duodenum, stomach) in the gastrointestinal tract. Appropriate insertion of feeding tube into the different anatomical regions required a preliminary study concerning the length of small intestines in dogs. Reviewing the literatute, we did not find any data on oral application of fat-containing infusions therefore in an other preliminary experiment we demonstrated the cholagogue effect of Lipofundin (Lipofundin MCT/LCT 20%, B. Braun Melsungen AG, Melsungen, Germany).

The Gastrointestinal Standardization Group of World Small Animal Veterinary Association (WSAVA) published an international standard for the histological evaluation of canine gastritis (Day et al., 2008), it's introduction in Hungary became inescapable.

In the second part of the dissertation an overall histopathological, clinicopathological and immunohistochemical evaluation of canine gastric biopsies obtained via gastroscopy and necropsy was performed. First of all we adapted the above mentioned WSAVA standard into the Hungarian veterinary practice. In our retrospective study the correlation between the

histopathological changes and clinical aspects was investigated. In a comparative immunohistochemical research the claudin expression of healthy gastric necropsy samples, lymphocytic gastritis and gastric signet-ring cell adenocarcinoma samples obtained via endoscopy was examined. Our goal was to find immunohistochemical markers for early detection of different pathological changes in canine gastric mucosa.

2. Effect of endoscopically guided feeding tube position and the place of feeding on gallbladder function in dogs.

2.1. Measurement of canine small intestines (anatomical preliminary study)

2.1.1. Material and methods

Our data were collected mostly on canine cadavers influenced by post mortem rigidity and autolysis, therefore it was important to compare this results with those obtained from alive animals. AIDR Auricoop Kft. (2120 Dunakeszi, Pálya u. 2.) made it possible on three Beagle dogs before and after euthanasia. In one of those three dogs a feeding tube was inserted into the duodenojejunal junction comparing the position of the tube with that of after euthanasia. All of the procedures were in accordance with the veterinary law and ethical regulations.

Groups of examined dogs:

1. different breed cadavers (n=25; 11 female, 14 male),
2. Beagle breed cadavers (n=10; 5 female, 5 male),
3. alive Beagle dogs (n=3; 2 female, 1 male).

Our examinations were performed at the Department of Anatomy and Histology (1. group), AIDR Auricoop Kft. (2. group), the Department and Clinic of Internal Medicine (3. group), radiological control at the Department and Clinic of Surgery and Ophthalmology. The post mortem examination of cadavers was performed at the Department of Pathology.

For the statistical analysis of the connection between the length of the small intestines and body weight or withers regression analysis was applied for determining the coefficient of determination (R^2).

2.1.2. Results and discussion

1. The length of small intestines is highly variable both in Beagle and other, different breed dogs.
2. Because of high variance of the data, a mathematical formula based on linear regression analysis can not be applied for calculating the length of small intestines according to the body weight or withers in dogs.

3. There is not any clinically relevant difference in the length of the small intestines measured in alive dogs or cadavers, however because of the small number statistical analysis was not performed.
4. It has been proved that the position of endoscopically guided feeding tube did not changed after the euthanasia controlled by abdominal radiography and necropsy.

2.2. Effect of Lipofundin meal on gallbladder function (preliminary study)

2.2.1. Material and method

Lipofundin was administered in 2 mL/kg doses in 7 Beagle dogs (gender: 3 male, 4 female; age: 10-22 months; body weight: 10-14.3 kg). To calculate the gallbladder volume the „area-length method" was used (Sterczer et al., 1996). The gallbladder volume was measured before the Lipofundin meal as a basic volume and after that every 10 minutes 3-3 times. The dogs were found healthy on the basis of clinical examination, blood laboratory results (differential blood cell count, quantitative blood cell count, total serum bile acid, alkaline phosphatase, alanine amino-transferase, gamma-glutamyltransferase, albumin, total protein and creatinine). They were kept in individual cages in the same room. Ultrasonographic examinations were performed after witholding the food for 24 hours.

For the statistical analysis the two-sample Student's t-test and R statistical software were used (R Development Core Team, 2006).

2.2.2. Results and discussion

Lipofundin induced the most pronounced reduction in gallbladder volume at 30 min in 5 and at 40 min in 2 dogs after its administration. The maximal gallbladder contraction was 44.2% (range: 35.3-57.6%). The volume of the gallbladder was reduced significantly between 10 and 50 min after Lipofundin administration. This rapid decline in volume was followed by gradual recovery of the gallbladder volume beginning at approximately 40 min. Oral application of the Lipofundin did not cause any side effects such as diarrhoea, vomiting or abdominal pain. In our research was proved the cholagogue effect of Lipofundin infusion applied orally. It is well tolerable, effective and easily applicable drug. The Lipofundin infusion proved to be useful for the examination of the effect of enteral feeding on gallbladder function in dogs.

2.3. Effect of endoscopically guided feeding tube position and the place of feeding on gallbladder function in dogs.

2.3.1. Material and method

Animals, measurement of gallbladder volume

Lipofundin was administered in 2 ml/kg doses in 5 Beagle dogs (gender: 3 male, 2 female; age: 32-36 months; body weight: 11.6-15.4 kg). To calculate the gallbladder volume the „area-length method" was used (Sterczer et al., 1996). The gallbladder volume was measured before the Lipofundin meal as a basic volume and after that every 5 minutes 3-3 times. The dogs were found healthy on the basis of clinical examination, blood laboratory results (differential blood cell count, quantitative blood cell count, total serum bile acid, alkaline phosphatase, alanine amino-transferase, gamma-glutamyltransferase, albumin, total protein and creatinine). They were kept in individual cages in the same room. Ultrasonographic examinations were performed after witholding the food for 24 hours.

Examination protocol

The examinations were performed in each dog on separate days after a 24-hour fasting period. The effect of Lipofundin meal on the gallbladder volume was examined in four anatomical positions (jejunum, duodenojejunal junction, descending duodenum, stomach). Under general anaesthesia the tube was introduced 20-30 cm into the jejunum by endoscopy (Pápa et al., 2009). After radiographic control 0.9% NaCl (normal saline) infusion was applied for 60 minutes at a rate of 2 mL/kg/min. The basic gallbladder volume was recorded by the ultrasound in the alert dogs. The Lipofundin meal (2 mL/kg) was applied through the tube for 15 minutes by syringe pump. The gallbladder volume was measured by ultrasound at 5-minute intervals for 60 minutes. Following that procedure normal saline (2 mL/kg/min) infusion was applied for 60 minutes. Under slight anaesthesia (propofol 3-6 mg/kg bolus IV) the tube was moved to the duodenojejunal junction under radiographic control and fixed to the skin of the nose and forehead. The above mentioned steps were repeated in each anatomical place (such as positioning of the tube under propofol anaesthesia, radiographic control, saline perfusion, determination of basic gallbladder volume, Lipofundin meal application, monitoring the gallbladder volume, saline perfusion). Finally the tube was removed under general anaesthesia. The duration of this procedure was about 10-12 hours.

On another day, after 24 hours of fasting the basic gallbladder volume of the alert dogs was measured by ultrasound, then 2 mL/kg Lipofundin infusion was given orally by syringe and the gallbladder volume was monitored for 60 minutes at 5-minute intervals.

Statistical analysis

For hypothesis testing, a linear mixed model was fit to the gallbladder volume change percentage data. The analysis was carried out in R 2. 10. 1 statistical software (R Development Core Team, 2009). The dependence between measurements from the same individual (dog) has been taken into account by modelling the within-dog error variance-covariance matrix. Linear mixed model was fit by Restricted Maximum Likelihood method (Pincheiro and Bates, 2000). Model effects were tested together based on their F values. All factors and potential interactions were evaluated with the cut-off for inclusion being $P < 0.05$. The position dependence of maximum volume change values was tested by ANOVA along with Tukey post hoc tests to detect significant differences.

2.3.2. Results and discussion

The Lipofundin meal applied through the feeding tube into the proximal 20-30 cm of the jejunum evoked the first significant gallbladder contraction (GBE1: mean 5.9%, range 2.6-9.6%) after 10 minutes perfusion ($P<0.05$). The mean maximal contraction was 7.2% (3.1-12.7%) achieved at 20 minutes ($P<0.05$). At the duodenojejunal junction the first significant gallbladder contraction (GBE2: mean 8.9%, range 3.2-12.3%) was already elicited at 5 minutes during the Lipofundin perfusion ($P<0.01$). The mean maximal contraction of the gallbladder was 12.9% (-0.9-22.1%) detected at 15 minutes ($P<0.001$). Lipofundin meal applied in the descending duodenum (GBE3) through the tube evoked the first significant gallbladder contraction at 5 minutes (mean 6.9%, range -2.4-11%) ($P<0.05$). The mean maximal contraction was 19.5% (range 8.3 to 27.3%) ($P<0.001$) achieved at 15 minutes. The orally applied Lipofundin infusion into the stomach elicited the first significant gallbladder contraction (GBE4) at 5 minutes at a mean value of 8.9% (1.9-21.3%) ($P<0.05$) and the mean maximal contraction was detected at 20 minutes at 30.4% (26.3-36.1%) ($P<0.001$).

The time and the magnitude of the maximal contractions evoked by Lipofundin in the investigated anatomical positions were different. The mean maximal contraction was 9.2% (range 3.7 to 13.8%) in the jejunum (GBE1), 16.6% (range 9.3 to 22.2%) in the duodenojejunal junction (GBE2), 26.2% (range 22.5 to 29.5%) in the descending duodenum (GBE3) and 34.3% (range 28.7 to 46.4%) in the stomach (GBE4). There were no significant

differences in maximal gallbladder contractions by Lipofundin applied into the jejunum and duodenojejunal junction (GBE1max/GBE2max) and between the descending duodenum and oral application into the stomach (GBE3max/GBE4max). However, the maximal response of the gallbladder to the Lipofundin meal was significantly different in the jejunum and duodenojejunal junction compared with the descending duodenum and stomach (GBE1-2max/GBE3-4max). There were no significant changes in gallbladder volume between the experiments during the normal saline infusion at the rate of 2 mL/kg/minutes.

In conclusion, we can establish that the deeper the position of the Lipofundin's application the smaller and shorter the evoked gallbladder contraction. This can be beneficial in biliary infections and obstructive disorders. According to our result Lipofundin applied into 20-30 cm deeply into the jejunum caused only week gallbladder contraction suggesting the usefulness of jejunal feeding tube placement canine pancreatitis. However this statement requires further examination.

3. Diagnostic role of endoscopy in canine gastric diseases

3.1. Retrospective analysis of canine gastric biopsies obtained via endoscopy

3.1.1. Material and methods

Origin of the samples

In our study gastric biopsy samples taken from canine vomiting patients coming for gastroscopy (SZIU Faculty of Veterinary Science, Small Animal Hospital, Endoscopic Laboratory of the Department of Internal Medicine) from January 2007 to December 2010 were investigated. Minimum 3-3 biopsy samples were obtained using endoscopic biopsy forceps from each anatomical region (fundus, corpus ventriculi, antrum pyloricum) of the stomach. The tissue samples were put in 8% neutral buffered formalin and marked.

The referred patients arrived from our University Clinic and from private veterinarians for the gastroscopy. In the investigation period 87 samples were taken from 81 dogs. Excluding the neoplastic and inadequate specimens, 66 samples were examined statistically from 60 dogs.

Histopathology

The histological evaluation was performed at the Department of Pathology at the Szent István University. The tissue samples were fixed at room temperature for 24 hours and embedded in paraffin. The 3-4 µm thick sections were routinely stained with haematoxylin-eosin for investigating the inflammatory and morphological abnormalities, and modified Giemsa and Warthin-Starry staines were applied for the detection of Gastric Helicobacter like Organisms (GHLO). The histological evaluation was in accordance with the standard of WSAVA (Day et al., 2008) that was modified with the fundic region of the stomach and GHLO examination.

Treatment

The grading classification of the applied treatment in chronological order:

1. symptomatic treatment (antiemetics, H2-blockers)
2. simple diet (intestinal formula of different brands or fat-restricted food without bones)

3. gluten free, novel protein diet
4. antibiotics (amoxicillin, enrofloxacin, other) or metronidazol for short term treatment
5. Helicobacterium eradication (amoxicillin-klavulanic acid 2 x 10 mg/kg/day for minimum 10 days, claritromycin 2 x 8-10 mg/kg/day, metronidazol 2 x 10-15 mg/kg/day treatment in combination),
6. prednisolone treatment (usually 2 mg/kg/day initial treatment, decreasing the dosage by 50% in case of efficiency ever 2 weeks).

The treatment of the patients was performed according to the above mentioned steps. If the patient did not improve, the next treatment protocol was used until to improvement.

Statistical analysis

Spearman-correlation was applied for examining the relationships (Reiczigel et al., 2010) using R 2.14.0 statistical software (R Development Core Team, 2011).

3.1.2. Results and discussion

Breeds that were over-represented included the mixed-breed 8 (13.3%), labrador retriever 4 (6.7%), yorkshire terrier 4 (6.7%), French bulldog 3 (5.0%) and golden retriever 3 (5.0%). During the evaluation of the histopathologic samples, lymphocytic-plasmacytic infiltrations 43 (65.2%), eosinophilic infiltrations 5 (7.6%), neutrophilic infiltrations 10 (15.2%), lymphoid follicular hyperplasia 6 (9.1%) and GHLO infection 34 (51.5%) were recorded. The necessary and sufficient therapeutic trial applied in the 66 cases included: 1. symptomatic treatment 12 (18.2%), 2. simple diet 9 (13.6%), 3. gluten free diet with novel protein source 17 (27.8%), 4. short-term antibiotic or metronidazole treatment 6 (9.1%), 5. Helicobacterium eradication 13 (19.7%), 6. prednisolone treatment 9 (13.6%).

According to the statistical analyzes in case of bitches, neutrophilic infiltration correlates positively (Spearman-correlation: 0.567, P=0.001), GHLO infection correlates negatively (Spearman-correlation: -0.39, P=0.03) with the age of the patients. The lymphocytic-plasmacytic infiltration correlates with GHLO infection negatively (Spearman-correlation: -0.39, P=0.03) in bitches and positively (Spearman-correlation: 0.44, P=0.007) in males. Neither the intensity of the inflammatory lesions (neutrophilic, eosinophilic, lymphocytic-plasmacytic) nor the level of GHLO positivity correlates with the applied treatment.

We can conclude that we adapted first an international standard for the histopathological evaluation of the gastric biopsies supplemented by the fundic region and GHLO infection in dogs. According our result we obtained valuable information in Hungarian dogs referred to gastroscopy about incidence of the GHLO infection and the different sort of gastritis.

A comprehensive study of canine gastric endoscopic biopsies including such a high number of cases has not been published yet.

The GHLO infection, the intensity of histopathological lesions, and the degree of applied treatment do not show clear correlations with each other in accordance with the literature.

3.2. Immunohistochemical examination of claudin-1, -2, -3, -4, -5, -6, -7, -8, -10 and -18 in canine healthy gastric necropsy samples, lymphocytic gastritis and gastric signet-ring cell adenocarcinoma samples

3.2.1. Material and method

Sampling, origin of gastric samples

Healthy gastric samples

The healthy gastric samples were collected by necropsy from the patients of the Small Animal Hospital at Szent István University died or euthanized due to non-gastrointestinal problems. Twenty adult dogs were examined during the study (age: 3-14 years, mean: 8 years; 9 females, 11 males). Tissue samples 2 x 2 x 0.5 cm in size were excised during necropsy from the fundus, body and antral region of the investigated fresh canine cadavers. The tissue samples were fixed in 8% buffered formalin solution at room temperature for 24 hours.

Lymphocytic gastritis samples

The claudin expression of gastroscopic biopsy samples of 12 dogs with lymphocytic gastritis was examined. The sample containing tissue blocks were selected from the collection of the Department of Pathology. Each sample was collected with gastroscopy between January 2007 and December 2013 in the Endoscopic Laboratory of the Department of Internal Medicine of the Faculty of Veterinary Science. In all cases 3-3 biopsy specimens were taken from the fundus, body and antral region of the examined stomach as it is mentioned in the retrospective study.

Signet-ring cell gastric adenocarcinoma

The claudin expression of gastroscopic biopsy samples of 14 dogs (age: 6,5-13,5 years; mean: 10 years; 4 female, 10 male) with signet-ring cell gastric adenocarcinoma was examined. The selection of samples was the same way as in case of lymphocytic gastritis samples.

Immunohistochemistry

Immunohistochemical detection of claudins was performed in formalin fixed, paraffin embedded sections. 3-4 µm thick sections were cut from each paraffin blocks, deparaffinised, rehydrated, and stained with antibodies to claudin-1, -2, -3, -4, -5, -6, -7, -8, -10, and -18. The slides were deparaffinised in xylene and graded ethanol. Prior to the application of the primary claudin antibodies at room temperature for 60 minutes, an appropriate antigen retrieval solution was used (Target Retrieval Solution, DAKO, Glostrup, Denmark, pH 6; microwave oven for 30 minutes). Immunohistochemical staining was performed with the streptavidin-peroxidase procedure (DAKO LSAB2 Kit). The chromogen substrate was 3, 3-diaminobenzidine (DAB). Mayer's haemalaun was used for counterstaining. In case of each claudin, appropriate tissue blocks were included for positive control, and the omittance of the primary antibody was used as negative control.

3.2.2. Results and discussion

In the case of healthy gastric mucosa and mild, moderate and severe lymphocytic gastritis intense, linear, membranous claudin-18 positivity was detected in the surface gastric cells and in the epithelial cells of the gastric glands both in the fundic and pyloric stomach regions. The mucous neck cells in the apical part of the glands, furthermore the parietal cells and chief cells of the basal part of the gland were all positive for claudin-18, in the same way as the enteroendocrine cells. Cells of the basal part of the pyloric glands showed intense, linear, membranous claudin-2 positivity in the case of mild, moderate and severe lymphocytic gastritis, but cells of the superficial portion of these glands, and the surface gastric cells in this region were claudin-2 negative. Fibroblasts, endothelial cells, lymphocytes of the propria layer, the smooth muscle cells and vegetative neurons were all negative for claudin-2. All gastric epithelial cells, the inflammatory cells, lymphocytes, plasma cells, and macrophages

were negative for claudin-1, -3, -4, -5, -6, -7, -8, and -10. The endothelial cells of the propria layer had intense claudin-5 positivity.

In gastric cancer at the invasive front the neoplastic cells originated from the epithelial cells of the fundic and pyloric glands were all negative for claudin-18. The dysplastic epithelial cells in the fundic glands showed week claudin-18 positivity compared with the surrounding normal gastric mucosa where the fundic surface gastric cells, the cells of the apical portion of the fundic gland such as mucous neck cells, and the cells of the basal part of the fundic gland including parietal cells, chief cells, furthermore enteroendocrine cells showed intense, linear, membranous claudin-18 positivity.

The neoplastic cells at the invasive front, and the dysplastic epithelial cells in the surrounding area were all negative for claudin-1, -3, -4, -5, -6, -7, -8 and -10.

According to our results claudin-18 is an important component of tight junction structure in the canine gastric mucosa. The expression of claudins in lymphocytic gastritis was similar to those in healthy canine gastric mucosa with no increased permeability detected at this level. The down-regulation of claudin-18 could be explained by early signs of tumorigenesis.

Our data suggest that down-regulation of claudin-18 may play an important role in canine gastric carcinogenesis, and especially in signet-ring cell gastric carcinoma it is associated with aggressive behavior of the tumor. This study demonstrated that the loss off claudin-18 is a novel diagnostic marker for canine signet-ring cell gastric carcinoma.

4. New scientific results

1. It can not be applied a mathematical formula based on linear regression analysis for calculating the exact length of small intestines according to the body weight or withers in dogs.
2. The cholagogue effect of orally applicated Lipofundin infusion is well measurable, thus it is useful for the examination of the gallbladder function influenced by the position of the enteral feeding tube. The deeper Lipofundin is applied into the small intestine the smaller and shorter gallbladder contraction was evoked. So we suspect that a feeding tube inserted in 20-30 cm deeply into the jejunum can be useful in the treatment in acute pancreatitis.
3. We adapted an international standard for the histopathologic evaluation of gastric biopsies supplemeted with GHLO examination. According our result we obtained valuable information on dogs from Hungary, referred to gastroscopy about the incidence of the GHLO infection and the different types of gastritis. A comprehensive study of canine gastric endoscopic biopsies including such a high number of cases has not been published in our country yet.
4. The claudin-18 is an important structural component of epithelial tight-junction structure presumably performing a paracellular barrier against gastric acid in the canine gastric mucosa similarly in mice.
5. The expression of claudins in lymphocytic gastritis was similar to those in healthy canine gastric mucosa with no increased permeability detected at this level.
6. In canine signet-ring cell gastric adenocarcinoma the claudin-18 expression of neoplastic cells at the invasive front can not be detected by the immunohistochemical method. The week claudin-18 positivity of the dysplastic epithel cells in the area surrounding the tumor could be an early diagnostic marker for tumorigenesis.

5. Possibilities for future research

Summarizing the results of the dissertation new questions arise in connection with the introduction of jejunal feeding tube in the therapy of canine acute pancreatitis and concerning the diagnostic and therapeutic consequences of gastric diseases. Those require prospective studies:

1. Early jejunal feeding in canine acute pancreatitis: prospective, randomized study.
2. Etiological approach of gastric disorders, endoscopic and histopathologic diagnosis, therapy, endoscopic control of recovery in a prospective study.
3. Follow-up of premalignant gastric diseases (atrophia, intestinal metaplasia, adenoma, MALT lymphoma) after Helicobacter eradication.
4. Screening examinations for Helicobacter reinfection in dogs.

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7. Scientific publications of the thesis

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3. **Psáder R.,** Pápa K., Hőnich E., Harnos A., Jakab Cs., Sterczer Á.: Kutyák endoszkópos módszerrel vett gyomorbioptátumainak retrospektív vizsgálata. Magy. Állatorv. Lapja, 2012. *134.* 537–548. **IF: 0,146**
4. **Psáder, R.,** Jakab, Cs., Máthé, Á., Balka, Gy., Pápa, K., Sterczer, Á.: Expression of claudins in the normal canine gastric musosa. Acta Vet. Hung., 2014. *62.* 13-21. **IF: *1,173**
5. **Psáder R.,** Jakab Cs., Balka Gy., Pápa K., Sterczer Á.: Lymphocytás gyomorgyulladásban szenvedő kutyák endoszkópos módszerrel vett gyomorbioptátumainak immunhisztokémiai vizsgálata klaudin-1, -2, -3, -4, -5, -6, -7, -8, -10 és -18 markerekkel. Magy. Állatorv. Lapja, 2014. *136.* 105–114. **IF: *0,146**

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