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Different solutions used for submucosal injection influenced early healing of gastric endoscopic mucosal resection in a preclinical study in experimental pigs

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Abstract
Background We hypothesised that different solutions for submucosal injection may influence early healing of endoscopic mucosal resection (EMR). The aim of this study was to evaluate histological and immunological changes after EMR in experimental pigs.
Materials and methods Two parallel EMRs on the anterior and posterior wall of the gastric body were performed by means of the cap technique in 21 female pigs. A glycerol-based solution (anterior EMR) and hydroxypropyl methylcellulose solution (posterior EMR) were applied for submucosal injection. The animals were sacrificed 7 days later, and tissue sections of all EMRs were stained using combined trichrome. Computer image analysis was used for objective evaluation of elastic and collagen fibres content. Two-colour indirect immunophenotyping of blood and gastric samples were performed using mouse anti-pig monoclonal antibodies.
Results The values of collagen fibre content 7 days after EMR were significantly higher in lesions after the use of solution A in comparison with solution B (2.10 ± 0.25% versus 1.57 ± 0.25%, $p = 0.009$). Concordant results were found in elastic fibres (3.23 ± 0.49% versus 2.93 ± 0.61%, $p = 0.018$). No systemic changes in major leukocyte subpopulations were found. In gastric tissue, lymphocyte subsets exhibited only minor changes. $CD4^+$ T-lymphocytes were increased in the healing tissue after EMR using solution A (17.08 ± 9.24% versus 9.76 ± 7.97%, $p = 0.011$). Significant increase of $SWC3^+$ leukocytes was observed after EMR using solution B (47.70 ± 25.41% versus 18.70 ± 12.16%, $p = 0.001$).
Conclusions The use of glycerol-based solution for submucosal injection was associated with more pronounced histological signs of early healing of EMRs compared with hydroxypropyl methylcellulose.

Keywords Submucosal injection · Healing · Gastric endoscopic mucosal resection · Experimental pigs

Endoscopic mucosal resection (EMR) has been widely used as a therapeutic method for resection of nonpolypoid lesions of the gastrointestinal tract, mostly of early cancer [1]. It was originally developed in Japan for the purpose of obtaining a larger biopsy specimen (strip-off biopsy) [2] and later revised to its current form [3, 4]. Most EMR techniques

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require use of submucosal injection to separate the lesion from the muscularis propria and thus reduce thermal injury and the risk of perforation and hemorrhage [1, 5–7].

In clinical endoscopy, numerous submucosal injection solutions have been proposed for EMR (such as saline solution with or without epinephrine, glucose, glycerol, hyaluronic acid or autologous blood) [1, 8–18]. However, no definitive proof of the superiority of any solution based on comparative studies has been provided and thus no conclusive recommendation for daily routine practice is available.

Preclinical studies in an experimental setting enable more detailed and precise evaluation as a paired arrangement of different solutions in a single animal is possible (to reduce interindividual variability), particular appraisal criteria can be set in advance and followed (i.e. histology, immunology, final outcome etc.) and defined variables can be exactly determined (i.e. different viscosity, electrical characteristics, injected volume etc.). In addition to this, the small adult pig can be used in experiments as an omnivorous representative due to its very similar gastrointestinal functions to man [19].

Previous studies of submucosal injection solutions for EMR have mostly evaluated ability to create a submucosal fluid cushion and its duration. Most authors think that sufficient duration of lesion lifting is more important for successful results of EMR than the types of solutions used themselves. However, only a limited amount of attention has been paid to the potential of injury to the muscularis propria. Possible tissue damage of the muscularis propria may result in impaired healing or even delayed complications (bleeding, perforation, stricture or stenosis) [20].

The overall objective of the present study was to evaluate the early histological and immunological markers of mucosal healing after gastric EMR in experimental pigs. We hypothesised that different composition of solutions of different viscosity used for submucosal injection could influence the early healing of EMR.

Materials and methods

Animals

Twenty-one mature female pigs (*Sus scrofa* f. domestica), hybrids of Czech White and Landrace breeds, weighing 34.1 ± 3.4 kg (4–5 months old), entered the study. They were kept in air-conditioned rooms (22 ± 2°C and 50 ± 10% relative humidity, with lights from 07:00 to 19:00 hours), fed twice a day (standard assorted food A1) and allowed access to water ad libitum. Handling of experimental animals was performed under the supervision of the Institutional Board of Animal Care of the Institute of Experimental Biopharmaceutics, Academy of Sciences of the Czech Republic.

Drugs

All endoscopies were carried out under general anaesthesia. Intramuscular injection of ketamine (20 mg/kg; Narkamon, Spofa, Praha, Czech Republic), azaperone (2 mg/kg; Stresnil, Janssen-Pharmaceutica, Beerse, Belgium) and atropine (0.05 mg/kg; Atropin, Biotika, Slovenská Ľupča, Slovakia) was used as an induction, continued by infusion of 1% thiopental (up to 25 mg/kg; Thiopental, Valeant Czech Pharma, Praha, Czech Republic) administrated to the lateral auricle vein.

Omeprazole (Zentiva, Praha, Czech Republic) was administrated as an i.v. bolus (40 mg) at the end of endoscopy to decrease the gastric production of hydrochloric acid and thus facilitate early healing of the base after EMRs.

For the pharmacological euthanasia i.v. administration of embutramide, mebezonium iodide and tetracaine hydrochloride (T61, Intervet International BV, Boxmeer, the Netherlands; dose of 2 mL/kg) was used.

Solutions for submucosal injection

Two different solutions were used for submucosal injection in each animal. Solution A contained 10% glycerol, 10% glucose and 0.1% Patent Blue V (Guerbet, Roissy, France); its viscosity was 1.69 mPa.s at 20°C. Solution B contained 0.85% hydroxypropyl methylcellulose in Ringer solution (Ivax Pharmaceuticals, Opava, Czech Republic), with a viscosity of 20.0 mPa.s at 20°C.

Endoscopy

All experiments were performed in the morning after overnight fasting in an aseptic operating room. Endoscopy procedures were performed using videogastroscopes GIF-Q130 (Olympus Optical Co, Tokyo, Japan) dedicated for animal use only. Two parallel standard EMRs on the anterior and posterior wall of the gastric body were performed by means of the cap technique in one session in all animals. Transparent soft caps with a diameter of 17.2 mm (MH 466, Olympus Optical Co, Tokyo, Japan) and EMR polypectomy snares (SD-221U-25, Olympus Optical Co, Tokyo, Japan) were used for EMRs. Solution A for the anterior EMRs and solution B for the posterior EMRs were applied; 10 mL of each solution was used for each submucosal injection. A 30-W seal cut (B Braun Aesculap, Melsungen, Germany) was used for all EMRs. Mucosa specimens were retrieved for subsequent histological and immunological investigation. Submucosal tattooing using a

suspension of highly purified carbon particles (Spot, GI Supply, Camp Hill, USA) was performed close (about 2 cm) to EMR with solution A on the anterior gastric wall. Each couple of EMRs in a single animal was performed by the same endoscopist (J.B. or M.K.). All endoscopies were video-recorded on digital versatile disk (DVD).

Animals were allowed food and water immediately after their recovery from general anaesthesia. Seven days later, the pigs were sacrificed by means of pharmacological euthanasia. Immediate autopsy was performed. Macrophotographs of the stomach with healing EMRs were taken and afterwards specimens of the gastric body were obtained at autopsy for subsequent analysis.

A 7-day interval was chosen based on results of a pilot testing EMRs performed previously in four animals with subsequent histological analysis of healing of EMRs on days 3, 5, 7, and 11.

Histology

The parts of gastric mucosa obtained by means of EMR (two samples from each animal) and specimens of gastric body at the area of previous EMR 7 days later (two samples from each animal) were carefully fixed with 10% neutral buffered formalin. Samples were subsequently embedded into paraffin, 1-μm-thick tissue sections were cut and Gram staining for bacteria as well as combined trichrome (green trichrome with Orange Q) staining were provided.

Image analysis

Stained samples were evaluated by using BX-51 microscope (Olympus Optical Co, Tokyo, Japan) and ImagePro 5.1 computer image analysis (Media Cybernetics, Bethesda, USA). Six microscopic fields with a size of 20,000 μm^2 at 600× original magnification were randomly selected from each sample for evaluation of elastic and collagen fibres content. The positive RGB structures were detected in the range: red, green, and blue for elastic fibres, where 0 is black and 255 is white as well as inverted RGB in the range: red, green, and blue detecting collagen fibres, where 0 is white and 255 is black. The percentage positivity of the viewing fields was subsequently measured. Percentage positivity means the percentage of positive objects.

Immunophenotyping of cells

Blood samples

Heparinised peripheral blood was obtained from vena cava cranialis just before EMR and 7 days later before pharmacological euthanasia in all experimental animals. Erythrocytes were removed using EasyLyse (Dako, Glostrup, Denmark) lysing solution according to the manufacturer's instructions. Remaining leukocytes were washed twice by means of centrifuging and resuspending the pellet in an ice-cold washing and staining buffer (WSB, phosphate-buffered saline containing 0.1% sodium azide and 0.02% gelatine from cold water fish skin, all reagents from Sigma-Aldrich, St. Louis, USA). Cells were counted and their density was set to 5×10^6 per mL.

Gastric samples

Gastric mucosa was obtained by means of EMR using either solution A or B. The samples from the healing lesions were excised at autopsy. Mucus was removed from the tissue by means of washing with phosphate-buffered saline and the cells from the superficial part of the tissue were released by gentle scraping using a scalpel; tissue aggregates were homogenised by pipetting. The suspension was then filtered through a fine nylon mesh and washed twice in ice-cold WSB, cells were counted and their density was set to 5×10^6 per mL. All samples were kept on ice until immunostaining.

Cell staining

Two-colour indirect immunophenotyping was performed as described elsewhere [21]. Briefly, 100 μL of the cell suspension (5×10^6 cells) was incubated for 30 min with a pair of optimally titrated mouse anti-pig monoclonal antibodies of different (sub)isotypes in 96-well microtitration plates, washed twice in WSB and Fc receptors on the cell surface were subsequently blocked by incubating the cells in WSB containing 10% heat inactivated nonimmune normal goat serum. After another centrifugation step, the binding of primary immunoreagents was visualised by resuspending the cells in WSB containing an appropriate cocktail of fluorescein isothiocyanate and phycoerythrin-conjugated goat anti-mouse polyclonal antisera specific for mouse Ig (sub)isotypes (Southern Biotech, Birmingham, USA). After 20 min of incubation the cells were washed twice in WSB. The panel of the following mouse anti-pig monoclonal antibodies was used: anti-CD45 (K252.1E4, IgG1), anti-CD2 (MSA4, IgG2a), anti-CD3ε (PPT3, IgG1), anti-CD4 (10.2H2, IgG2b), anti-CD8 (76-2-11, IgG2a), anti-TCR$\gamma\delta$ (PPT16, IgG2b), anti-SWC3a (74-22-15, IgG2b) and anti-IgM (Lig4, IgG1).

Flow cytometry

The samples were acquired on a CyAn ADP analyser (Dako, Glostrup, Denmark) and 30,000–500,000 events were collected in each sample depending on leukocyte proportions. Damaged and dead cells were excluded from

analysis using propidium iodide (10 μL/mL) fluorescence. Data analysis was performed using Summit 4.3 software (Dako, Glostrup, Denmark).

Statistical analysis

Data were statistically analysed with paired *t*-test and Mann–Whitney rank-sum test. The second-type error β was calculated when appropriate. Statistical software used for these analyses was SigmaStat version 3.1 (Jandel Corp, Erkrath, Germany). A *p*-value of less than 0.05 was accepted as statistically significant.

Ethics

The study was approved by the Institutional Review Board of Animal Care Committee of the Institute of Experimental Biopharmaceutics, Academy of Sciences of the Czech Republic, Protocol Number 149/2006. Animals were held and treated in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [22].

Results

Histology and image analysis

There was no gross complication of the EMR itself in any animal (Fig. 1). No presence of bacterial infection in any sample (as a complication of EMR) was found in histology.

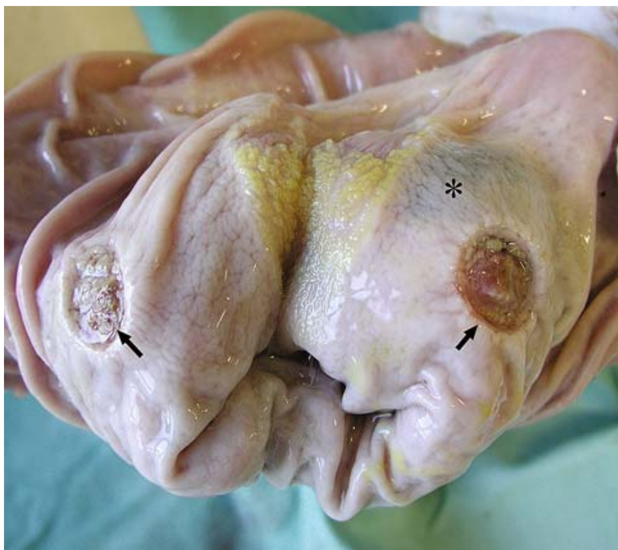

Fig. 1 Representative view of two healing lesions 7 days after EMRs. The posterior wall is on the left and the anterior one on the right. Submucosal tattooing by suspension of highly purified carbon particles is marked by an asterisk

The results of quantitative histological detection of combined trichrome staining of granulous tissue in the healing lesions of the gastric wall after EMRs are shown in Table 1. The values of elastic fibre content 7 days after EMR were significantly higher in lesions after the use of solution A in comparison with after use of solution B (Figs. 2 and 3). The same results were observed during measuring of content of collagen fibres. Lesions after EMR using solution A expressed a higher percentage of collagen fibres than those having used the solution B for submucosal injection before EMRs (Figs. 4 and 5).

Leukocyte population and lymphocyte subsets analysis

No systemic changes in major leukocyte populations, lymphocyte subpopulations and their subsets were found during the experiment. As typical for an outbred population, individual pigs differed in the proportions of major lymphocyte subpopulations and subsets in peripheral blood and the gastric EMR did not cause any significant changes or trends in circulation. Well-defined lymphocyte subsets such as $CD4^+CD8^+$ double-positive T cells, $CD4^-CD8^+$ and $CD4^+CD8^-$ cytotoxic and helper/regulatory $\alpha\beta$ T cells, respectively, $CD3^-CD8^+$ NK cells, $TCR\gamma\delta^+$ T cells

Table 1 Content of collagen and elastic fibres in granulous tissue 7 days after EMR, in percent mean ± standard error on the mean (SEM)

Parameter	Solution A	Solution B	Significance
Collagen fibres	2.10 ± 0.25	1.57 ± 0.25	$p = 0.009$*
Elastic fibres	3.23 ± 0.49	2.93 ± 0.61	$p = 0.018$*

* Mann–Whitney rank-sum test

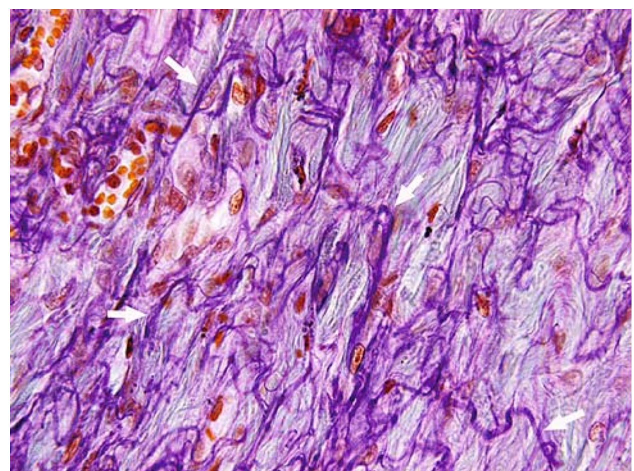

Fig. 2 Detailed view of granulous tissue (magnification 600×) 7 days after EMR in the stomach (solution A). Elastic fibres are bright violet (arrows). Combined trichrome (green trichrome with Orange Q) staining

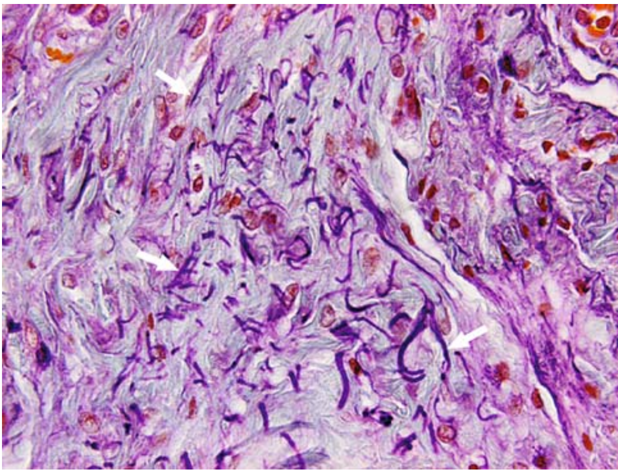

Fig. 3 Detailed view of granulous tissue (magnification 600×) 7 days after EMR in the stomach (solution B). Elastic fibres are bright violet (arrows). Combined trichrome (green trichrome with Orange Q) staining

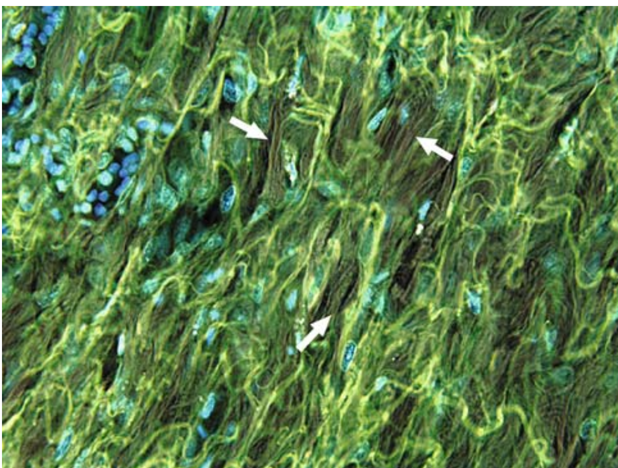

Fig. 4 Inverted image of Fig. 2, demonstrating numerous collagen fibres in dark brown (arrows)

or B cell subsets differing in CD2 expression exhibited only minor changes typical for different collection days in individual pigs (data not shown). Similar to lymphocytes, analysis of myelomonocytic cells in peripheral blood did not reveal any reproducible changes before and 7 days after EMR.

In gastric samples, the $CD4^+$ single-positive T-cells appeared to increase their relative numbers in the healing wound 7 days after the submucosal injection of solution A ($9.76 \pm 7.97\%$ versus $17.08 \pm 9.24\%, p = 0.011$). In tissue samples, a significant increase of the proportion of $SWC3^+$ leukocytes was observed on day 7 (compared with day 1) after the submucosal injection of solution B ($18.70 \pm 12.16\%$ versus $47.70 \pm 25.41\%, p = 0.001$). Results with solution A appeared to have a similar trend but the difference between day 1 and 7 was not statistically significant. There was no

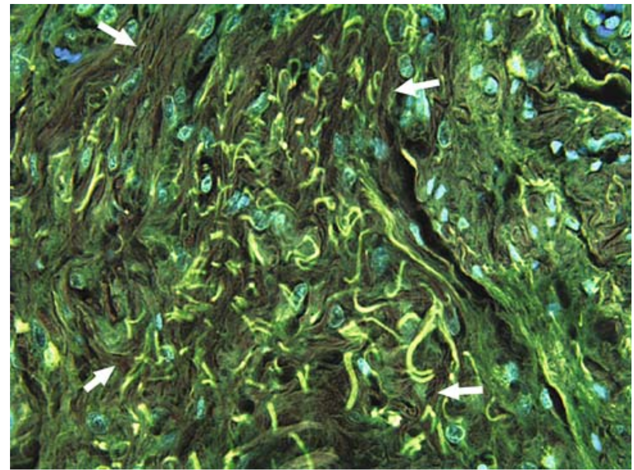

Fig. 5 Inverted image of Fig. 3, demonstrating numerous collagen fibers in dark brown (arrows)

significant difference in the proportion of $SWC3^+$ leukocytes on day 7 after the use of solutions A or B ($p = 0.129$; power of the performed test 0.198). Scatter characteristics of the myelomonocytic ($SWC3^+$) population that enlarged its size after EMR suggested that such cells were granulocytes (high side scatter values).

Overall healing characteristics

The results of particular parameters displayed above clearly show that different solutions used for submucosal injection before EMR influenced early signs of healing assessed by histology. The use of the glycerol-based solution was associated with more pronounced histological signs of early healing compared with the solution of hydroxypropyl methylcellulose. There were no significant changes of major leukocyte populations, lymphocyte sub-populations and myelomonocytic cells in peripheral blood as a systemic response 7 days after EMRs. The increased myelomonocytic population in the healing lesion after EMR were granulocytes.

Discussion

In this study, we compared different solutions used for submucosal injection and evaluated their impact on early mucosal healing after gastric EMR in experimental pigs. The use of the glycerol-based solution was associated with more pronounced histological signs of early healing compared with the solution of hydroxypropyl methylcellulose. Both solutions are highly hydrophilic but they markedly differed in their viscosity. However, the possible beneficial mechanism of glycerol-based solution in the early stage of healing after EMR remains unclear. Uraoka et al. [12]

compared glycerol and saline solution for EMR in patients with colorectal laterally spreading tumours. Glycerol provided a better resection rate but there was only a positive trend to parallel lower local recurrence rate (statistically not significant) [12]. Feitoza et al. [23] tested hydroxypropyl methylcellulose in experimental pigs. Hydroxypropyl methylcellulose was injected into the oesophageal submucosa (no subsequent EMRs were done). Histologic findings were fully normal only at 6% of the sites. Histology demonstrated negligible alterations in 33 of 36 (92 %) places, including the presence of a minimal amount of residual hydroxypropyl methylcellulose [23]. As no EMR was performed, this study could be compared only partly with our experiment. Nevertheless, deeper injection could administrate some hydroxypropyl methylcellulose into the muscular layer or even to the adventitia/serosa.

Mucosal wound healing is a complex process. The initial stage of healing within the first 24 h is characterised mostly by substantial infiltrate of neutrophils. By days 3–5, neutrophils have been largely replaced by monocyte–macrophage lines and fibroblasts, and granulation tissue progressively invades the healing area. Migration and proliferation of both parenchymal and connective tissue cells have been augmented [24]. Adequate reconstitution requires the coordinated interaction of endothelial cells and fibroblasts during the proliferation phase of healing. Endothelial cells assure neoangiogenesis, fibroblasts fill the defect and provide extracellular matrix proteins, and myofibroblasts are believed to support the reconstitution of microvessels [25]. The extracellular matrix is constituted by collagen and elastic fibres, proteoglycans (heparan sulphates), adhesive glycoproteins (fibronectin) and integrins. By the end of the first week, there is continued collagen accumulation and fibroblast proliferation. The leukocyte infiltrate and increased vascularity are substantially diminished [24]. At this stage, collagen and elastic fibres are the most relevant markers of healing. In our study 1 week after EMR, we found a significantly higher content of both collagen and elastic fibres in healing lesions having used solution A (glycerol-based) for submucosal injection for EMR compared with solution B (containing hydroxypropyl methylcellulose). This exact assessment was made possible owing to objective image analysis using computer software. Such an evaluation would not be conceivable with the naked eye. Both EMR lesions were of the same size, made in the gastric body during the same session in all animals, and thus fully comparable.

Studies in healing of gastric ulcers showed that extracellular matrix components (procollagens, collagen type I, III and IV, laminin, fibronectin) play an important role in attachment, migration and repair of wounded cultured gastric epithelial cells [26], in experimental animals [27] and in healing of gastric ulcers in humans [28, 29].

The leukocyte compartment in the blood and gastric tissue at the site of treatment was determined by double colour immunophenotyping and flow cytometry at the time of the submucosal injection of particular solutions for EMR (either A or B) and 7 days later. The samples of gastric mucosa obtained by means of EMR and healing lesions collected at autopsy 7 days later were histologically different. While gastric samples from the beginning of the experiment mostly consisted of undamaged layer of mucosa containing only a few intraepithelial lymphocytes and a population of lamina propria lymphocytes with TCR$\alpha\beta^+$ T-cells and TCR$\gamma\delta^+$ T cells possessing the CD4$^+$ and/or CD8$^+$ surface phenotype and some NK cells [30], the healing gastric lesion 7 days after EMR was infiltrated by increased numbers of myelomonocytic cells in both samples, which corresponded to the healing process within the mucosal layer. The slightly increased relative numbers of CD4$^+$ single-positive T-cells at the site of EMR 7 days after the endoscopy may reflect the recruitment from circulation of helper/regulatory T lymphocytes with a possible role in regulating the healing process via the cytokine network. The accumulation of myelomonocytic cells within the damaged tissue is a typical example of monocyte and granulocyte recruitment during the inflammatory and healing process, where such cells play an important role in the tissue repair process [31]. In our setting, there was no significant difference in the proportion of SWC3$^+$ leukocytes in the healing lesion after EMRs on day 7 between the use of solutions A and B for submucosal injection. However, the power of the performed test (0.198) was below the desired power of 0.800. This could indicate that we were more likely not to detect a difference when one actually existed. This is why it is necessary to be cautions in overinterpreting the lack of difference found here. We hypothesise that the difference could be identified some time earlier. Lower proportions of B cells in the gastric samples (less than 5% of lymphocytes when compared with the circulation where typically more than 10% of sIgM$^+$ lymphoid cells were present) is typical for lymphocyte subset composition of suspension isolated from nonlymphatic tissues.

One possible limitation of our current study is that it did not deal with the final outcome of EMR. Kamler et al. [32] performed circumferential oesophageal EMRs in experimental pigs. Saline solution was used for submucosal injection. Oesophageal strictures developed in three of eight animals over 3–5 weeks [32]. Thus we can speculate whether different solutions for submucosal injection would influence not only early healing but also final outcome of EMR. However, in the early stage of wound healing (within the first 14 days), presence of collagen (together with elastic fibres and granulous tissue) is not related to scar formation. At this stage, collagen and elastic fibres are

the most relevant markers of healing. Last but not least, we do not think that blood supply would influence the rate of healing (i.e. content of collagen and elastic fibres). The porcine gastric fundus is larger compared to the human one, supplied mostly by the left gastric artery [33]. However, there is no difference in blood supply of the anterior and posterior walls of the porcine gastric body [34, 35], accounting for half of total gastric blood flow [36]. The fundus and gastric body can be easily recognised and distinguished at gastroscopy with a clear sharp borderline (mucosa of the gastric fundus is markedly darker). All EMRs in our study were placed in the central part of the gastric body.

In conclusion, both glycerol- and hydroxypropylmethylcellulose-based solutions provided optimal lifting of the mucosa before EMRs. The use of glycerol-based solution for submucosal injection was associated with more distinct signs of early healing in experimental pigs.

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