1 **Title**

2 **Sprayable gelatin microparticles prevent delayed gastric bleeding in an anticoagulated**

3 **swine model**

4

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38 Tanaka, Shiho Arima, Shinichi Hashimoto, Hisashi Sahara, and Shuji Kanmura; final approval

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Abstract

Background: Delayed bleeding following endoscopic mucosal resection or endoscopic submucosal dissection is a serious concern in patients receiving anticoagulant therapy. We previously established an experimental animal model of delayed gastric bleeding following endoscopic resection under controlled anticoagulation. In this study, we aimed to evaluate the potential preventive effect of hydrophobized microparticles (hMPs), a novel sprayable wound-covering material derived from Alaska pollock gelatin, in preventing delayed bleeding.

Methods: Twelve gastric mucosal defects were created by endoscopic mucosal resection with ligation in three CLAWN miniature swine. To induce delayed bleeding, systemic heparin was administered after confirming an activated clotting time greater than 220 s. hMPs were endoscopically sprayed into each ulcer base (200 mg/ulcer). Endoscopic examination was performed 24 h later. The primary endpoint was the presence or absence of delayed bleeding. Secondary endpoints included hMP retention, ulcer coverage rate, presence of exposed vessels, and vascular coverage by hMPs.

Results: During the 24-hour observation period, no delayed bleeding was observed in any of the three CLAWN miniature swine treated with hMPs. Hemoglobin levels on Day 1 remained stable in all swine. hMPs were present in 100% of the ulcers. Complete ulcer base coverage (100%) was achieved in 55.6% (20/36) of the sections, with ≥50% coverage observed in all sections. Exposed vessels were identified in 36.1% (13/36) of ulcers, and all were covered with

61 hMPs.

62 ***Conclusion*:** Endoscopic spray application of hMPs demonstrated strong adhesion to gastric

63 ulcers and was associated with the absence of delayed bleeding in this short-term experimental

64 model. These findings suggest that hMPs may represent a promising strategy for reducing the

65 risk of delayed bleeding after gastrointestinal endoscopic surgery.

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Introduction

Endoscopic submucosal dissection (ESD) and endoscopic mucosal resection (EMR) are well-established treatments for early gastric tumors [1]. Delayed bleeding occurs in approximately 1.8–15.6% of patients after ESD [2,3], with antithrombotic therapy recognized as a significant risk factor. According to a recently proposed predictive model from Japan, the incidence of delayed bleeding ranges from 2.8% in the low-risk group to 29.7% in the very-high-risk group [4]. With an aging population, the number of patients receiving antithrombotic therapy continues to increase [5], making delayed bleeding after ESD an increasingly important clinical concern.

Despite its clinical importance, no definitive strategy has been established for preventing delayed bleeding after ESD [6]. A major limitation in developing such interventions is the lack of a reliable animal model for delayed post-ESD bleeding. We recently established such a model using gastric mucosal resection with systemic anticoagulation via a single bolus of heparin followed by continuous infusion [7]. We developed a sprayable wound dressing comprising multifunctional hydrophobized microparticles (hMPs) derived from swine gelatin [7]. These particles demonstrate strong tissue adhesion, even in wet environments, and effectively suppress submucosal fibrosis in the post-ESD setting [7,8]. More recently, we developed hMPs using fine particles derived from Alaska pollock gelatin, which maintained

88   strong adhesive properties [9,10]. These hMPs have shown efficacy in closing gastrointestinal

89   perforations, reducing inflammation in duodenal ESD ulcers [11], and preventing esophageal

90   strictures in animal models [12].

91   In this study, we aimed to evaluate the efficacy of hMPs, a novel sprayable wound-covering

92   material derived from Alaska pollock gelatin, in preventing delayed bleeding.

93

94   **Materials and Methods**

95   ***Experimental animals***

96   Three CLAWN miniature swine (age: 6 months; weight: 14–17 kg; procured from Kagoshima

97   Miniature Swine Research Center, Kagoshima, Japan) were used in this study. As

98   premedication, the animals were intramuscularly injected with ketamine (15 mg/kg; Daiichi

99   Sankyo Propharma Co., Ltd., Tokyo, Japan) or xylazine (2 mg/kg; Bayer Yakuhin, Ltd., Osaka,

100   Japan). An endotracheal tube (Smith Medical Japan, Tokyo, Japan) was inserted, and anesthesia

101   was maintained with inhaled isoflurane (1.5–3.0%) (DS Pharma Animal Health Co., Ltd.,

102   Osaka, Japan), delivered via a ventilator, with continuous monitoring of heart rate, respiratory

103   status, and oxygen saturation throughout the procedure.

104   ***Endoscopic mucosal resection with ligation (EMR-L) procedure and post-procedure follow-***

105   ***up***

106   An upper gastrointestinal endoscope was orally inserted into each swine to create gastric

7

107   mucosal defects using the EMR-L technique [13]. Twelve artificial gastric ulcers were created

108   by an endoscopist by performing the EMR-L technique using an upper gastrointestinal

109   endoscope (GIF-Q260J; Olympus, Tokyo, Japan), and a videoscope system (EVIS LUCERA

110   CV-260SL; Olympus) was used to ensure consistent collection of data on delayed bleeding.

111   Markings were made at 12 locations: lesser curvature, anterior and posterior walls of the upper

112   and middle gastric bodies, gastric angle, and lower gastric body. A polypectomy snare

113   (Captivator™ II 15 mm; Boston Scientific, USA) in coagulation mode was used for marking.

114   One milliliter of 0.9% saline containing indigo carmine (Otsuka Pharmaceutical Co. Ltd.,

115   Tokyo, Japan) was injected into the submucosa using an injection needle to achieve adequate

116   lifting. Endoscopic variceal ligation (EVL) was performed using a ligation device (Pneumo

117   Activate EVL; Sumitomo Bakelite, Tokyo, Japan), followed by snaring and resection with a

118   high-frequency device (Pulse-Cut Fast mode, 120 W, ESG-100; Olympus) [13,14]. All EMR-

119   L procedures were performed by S.U. Solid food was withheld from the day before the

120   procedure until the day after; however, water was provided ad libitum. No proton pump

121   inhibitor was administered following EMR-L [7]. Feeding was managed by a specialized

122   animal technician at the Kagoshima University Animal Experimental Facility, and the animals'

123   conditions were monitored daily. Animal care, housing, and surgical procedures were

124   conducted in compliance with the Kagoshima University Animal Experiment Committee

125   guidelines. Necropsies were performed by several researchers in accordance with the ethical

126   guidelines of the Kagoshima University Animal Experimentation Facility. This study was

127   approved by the Animal Experiment Committee of Kagoshima University (approval number:

128   MD23056).

129   This study is reported in accordance with the ARRIVE guidelines.

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131   ***hMPs***

132   Hydrophobically modified Alaska pollock gelatin was synthesized via the reaction between a

133   primary amine and a decanoic anhydride [15]. The hMPs were prepared using a coacervation

134   method in a water/ethanol mixed solvent [16]. Optimization of the alkyl chain length (decanoyl

135   groups, C10) and the degree of substitution (50 mol% of amino groups in Alaska pollock

136   gelatin) enhanced the mechanical strength of the hydrogel formed by the hydration and fusion

137   of the microparticles. Scanning electron microscopy confirmed that the resulting hMPs were

138   microparticles (Figure 1).

139   ***Delivery of hMPs***

140   The hMPs (200 mg) were packed into small vials. A battery-powered endoscopic injector (Alto

141   Shooter®, Kaigen, Tokyo, Japan), designed for the application of powdered agents, was used

142   to spray the hMPs. The vial was directly attached to the Alto Shooter®, and the powder was

143   sprayed onto the injured mucosa through the endoscope channel after removing the nozzle.

144   Overall, 200 mg hMPs (one vial) were applied to each ulcer (Figure 2).

145 *Heparin administration, activated clotting time (ACT) measurement, and swine dissection*

146 All animals received heparin via a catheter inserted into the external jugular vein. Catheters

147 (Argyle Fukuroi; CV catheter, 14 cm × 30 cm; Cardinal Health, USA) were inserted for blood

148 sampling and heparin administration [7]. The experimental protocol is illustrated in Figure 3.

149 After EMR-L, 50 U/kg unfractionated heparin (heparin sodium; Mochida Pharmaceutical Co.,

150 Ltd., Tokyo, Japan) was administered intravenously. The ACT was measured 10 min later using

151 a coagulation analyzer (Hemochron Jr Signature+, Accriva Diagnostics, Inc., USA). Additional

152 doses of 50 U/kg were administered every 10 min until the ACT exceeded 220 s, following a

153 previously established protocol [7]. Continuous heparin infusion (50 U/kg/h) was then initiated

154 using a portable disposable infusion pump (SUREFUSER® A, SFS-1002D, flow rate 2.1 mL/h;

155 NIPRO, Osaka, Japan). ACT was monitored at 0.5, 1, 2, and 4 h after starting the continuous

156 infusion. Endoscopic observation was performed 24 h after EMR-L to assess delayed bleeding.

157 The animals were then euthanized with an intravenous injection of thiamylal sodium

158 (ISOZOL®, Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan; 90 mg/kg) and potassium

159 chloride (Terumo Corporation, Tokyo, Japan; 20 mEq), followed by abdominal dissection and

160 removal of the stomach.

161 *Humane endpoints and animal welfare monitoring*

162 All animals were monitored at least twice daily by trained animal technicians for activity level,

163 respiratory pattern, posture, and signs of bleeding. Humane endpoints were predefined as

164 follows: (1) persistent inactivity despite stimulation, (2) signs of respiratory distress, and (3)

165 marked deterioration in physiological status. Animals meeting any of these criteria were

166 humanely euthanized immediately to minimize suffering. No unplanned humane euthanasia

167 was required during the study.

168

169 *Histological analyses*

170 Tissue specimens were fixed in 10% neutral-buffered formalin (Kenei Pharmaceutical, Osaka,

171 Japan) for 48 h, sectioned into 20-mm squares, embedded in paraffin, sliced into 2-µm-thick

172 sections, and stained using hematoxylin and eosin or Masson's trichrome. The retention and

173 coverage of hMPs, the presence of exposed vessels at the ulcer base, and the extent of hMPs

174 adherence to these vessels were assessed histologically.

175 *Outcome measures*

176 The primary endpoint was the presence or absence of delayed bleeding [7]. Hematemesis was

177 assessed on the day after the procedure, and melena was evaluated during necropsy. Delayed

178 bleeding was diagnosed if at least one of the following criteria was met: (1) hematemesis or

179 vomiting of blood within 24 h after the procedure, or (2) presence of blood clots or retained

180 blood in the stomach observed via endoscopy within 24 h [7]. Hemoglobin (Hb) levels were

181 measured immediately after catheter insertion and again between anesthesia induction and

182 endoscopic observation the next day, and the values were compared.

183   Secondary endpoints included the retention and coverage rates of hMPs at ulcer sites (n = 36),

184   the presence of exposed vessels at the ulcer base, and the proportion of hMPs adherent to those

185   vessels. The coverage rate was defined as the percentage of the ulcer base length covered by

186   hMPs, as assessed on the bisecting plane of the ulcer.

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188

189   **Results**

190   The characteristics and findings for each swine are presented in Table 1. All 12 ulcers had an

191   approximate diameter of 10 mm. The size of the ulcer was measured using a major forceps.

192   The submucosal layer remained intact at all sites, and the muscularis propria was identifiable.

193   No perforations or active bleeding were observed.

194   The time course of ACT values following heparin administration is shown in Figure 4. Heparin

195   (50 U/kg) was repeatedly administered until the ACT exceeded 220 s in all the swine. The ACT

196   values before continuous infusion were 265, 281, and 236 s in the first, second, and third swine,

197   respectively. The maximum recorded ACT values were 310, 347, and 240 s, respectively.

198   No delayed bleeding was observed in any swine treated with hMPs (Figure 5). Hb levels were

199   10.4 ± 1.0 g/dL at baseline and 11.5 ± 1.2 g/dL after treatment. The mean change was 1.1 ± 1.8

200   g/dL. The Hb levels remained stable in all swine （Before EMR-L: 10.4 ± 1.0 g/dL, After EMR-

201   L: 11.5 ± 1.2 g/dL). hMPs were detected in all 36 ulcer sections, with a 100% retention rate.

202 Complete coverage (100%) of the ulcer base was observed in 55.6% (20/36); 75–99%, in

203 19.4% (7/36); and 50–74%, in 25.0% (9/36); all ulcers exhibited ≥ 50% coverage (Figure 6).

204 Exposed vessels were identified in 36.1% (13/36) of the ulcer bases, and all were covered by

205 hMPs. Representative histological images are shown in Figure 7. hMPs were firmly attached

206 to the ulcer base and provided additional coverage over the exposed vessels.

207

208 **Discussion**

209 In this study, we evaluated the preventive efficacy of hMPs derived from Alaska pollock gelatin

210 using a newly established animal model of delayed bleeding after endoscopic treatment. Under

211 the conditions of this study, no delayed bleeding was observed, and relatively high ulcer

212 coverage rates as well as stable adherence to exposed vessels were confirmed, suggesting that

213 hMPs may contribute to the prevention of delayed bleeding in an anticoagulated swine model.

214 Delayed bleeding following endoscopic procedures is a serious adverse event in patients

215 receiving antithrombotic therapy [2,3]. Various preventive strategies, including mechanical

216 closure and the use of fibrin sealants, have been investigated [17-20].The hMPs used in this

217 study demonstrated strong adhesive properties even in wet environments, which is consistent

218 with the previous success of gelatin-based derivatives in ulcer protection and stricture

219 prevention, supporting their potential applicability as a wound-covering material in the

220 gastrointestinal tract.

221 A notable strength of this study is that it is the first report of a preventive intervention against

222 delayed bleeding in our newly developed [7], reproducible post-endoscopic bleeding animal

223 model. This model provides a valuable platform for the preclinical evaluation of various

224 hemostatic and wound-covering technologies.

225 We hypothesized that hMPs prevent delayed bleeding by firmly adhering to the ulcer base and

226 shielding exposed vessels from chemical and mechanical insults such as gastric acid and food

227 residue. Histological evaluation confirmed that the hMPs adhered to and covered the exposed

228 vessels. Thus, the endoscopic application of hMPs may effectively prevent delayed bleeding

229 following gastric mucosal resection (Figure 8).

230 The clinical significance of our findings lies in their potential to offer a novel prophylactic

231 strategy for high-risk patients after gastric ESD, including those receiving anticoagulant

232 therapy. If implemented in clinical practice, this technology could not only reduce the risk of

233 bleeding but also decrease the procedural workload for physicians and offer economic benefits

234 by shortening hospital stays and avoiding readmissions. Given the aging population and the

235 increasing prevalence of antithrombotic medication use, hMPs represent a promising and

236 practical option for clinical implementation. This study has some limitations. First, the

237 observation period was limited to 24 hours after EMR-L. Although this duration is relatively

238 short for fully evaluating delayed bleeding, previous clinical studies have reported that a

239 substantial proportion of post-ESD or post-EMR bleeding events occur within the first 24 hours,

240   particularly in patients receiving antithrombotic therapy [21,22].Therefore, evaluation during

241   this early phase remains clinically relevant; however, longer-term observation will be required

242   in future studies. Second, this was a preclinical animal study involving only three CLAWN

243   miniature swine, which limited the statistical power and generalizability of the findings.

244   Therefore, these results should be interpreted with caution. Third, no concurrent control group

245   was included. In a previously established swine model using a similar anticoagulation protocol,

246   delayed bleeding occurred in 75% (3 of 4) of swine when continuous heparin infusion was

247   initiated at an ACT threshold of ≥200 seconds, and in all swine when initiated at an ACT

248   threshold of ≥220 seconds [7]. The present study employed the latter, stricter anticoagulation

249   condition, suggesting that this model represents a high-risk setting for delayed bleeding.

250   Although these findings support the feasibility of the observed preventive effect, direct

251   comparison with a concurrent control group would further strengthen the evidence. Fourth, the

252   in vivo retention time of hMPs could not be fully evaluated because all animals were euthanized

253   24 h after EMR-L. Although hMPs were present at that time, their long-term persistence

254   remains unclear. In addition, the effects of human gastric peristalsis and the digestive

255   environment on hMPs retention require further investigation. Furthermore, mucosal resection

256   was performed using the EMR-L method rather than the ESD; therefore, its usefulness in

257   preventing post-ESD bleeding remains unknown. However, as EMR-L removes almost the

258   entire submucosal layer, similar to ESD, and multiple areas, it may also be useful for preventing

259 post-ESD bleeding. Finally, the gelatin microparticles used in this study are not yet

260 commercially available, and precise cost evaluation is therefore difficult at this stage.

261 Manufacturing costs and cost-effectiveness compared with existing hemostatic strategies will

262 be important considerations for future clinical translation.

263 Collectively, these limitations indicate that our work represents an initial proof-of-concept

264 study demonstrating the feasibility and potential efficacy of sprayable hMPs in preventing

265 delayed bleeding. Future studies should ideally include larger sample sizes and, ultimately,

266 multicenter clinical trials to assess safety and efficacy, along with investigations exploring its

267 applicability to other gastrointestinal sites and broader high-risk populations.

268 Endoscopic application of hMPs on mucosal defects resulted in strong tissue adherence and

269 was associated with the absence of delayed bleeding in this high-risk animal model. These

270 findings suggest that hMPs may represent a promising prophylactic strategy for managing post-

271 endoscopic bleeding, particularly in patients receiving anticoagulant therapy.

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278 **Author contributions**

279 Conception and design: Fumisato Sasaki; data analysis and interpretation: Shohei Uehara and

280 Fumisato Sasaki; drafting of the article: Shohei Uehara and Fumisato Sasaki; critical revision

281 of the article for important intellectual content: Hidehito Maeda, Makoto Hinokuchi, Akihito

282 Tanaka, Shiho Arima, Shinichi Hashimoto, Hisashi Sahara, and Shuji Kanmura; final approval

283 of the article: all authors.

284 Shohei Uehara and Fumisato Sasaki contributed equally to this study and share first authorship.

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287 **Conflict of interest**

288 Shohei Uehara, Fumisato Sasaki, Hidehito Maeda, Makoto Hinokuchi, Akihito Tanaka, Shiho

289 Arima, Shinichi Hashimoto, Shuji Kanmura, Hisashi Sahara, Yasuko Kobayashi, Akihito

290 Nishiguchi and Tetsushi Taguchi declare no conflict of interest for this article.

291

292 ▪ Informed Consent: N/A

293 ▪ Registry and the Registration No. of the study/trial: N/A.

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294 ▪ Animal Studies: Animal care, housing, and surgical procedures were conducted in

295   compliance with the Kagoshima University Animal Experiment Committee guidelines.

296   Necropsies were performed by several researchers in accordance with the ethical

297   guidelines of the Kagoshima University Animal Experimentation Facility.

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299 **Data availability**

300 All processed data supporting the findings of this study are included in this published article

301 and its supplementary information files. The raw image data are not publicly available due to

302 file size limitations, but are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1. Morphology and appearance of the hMPs

405 Left: SEM image of hMPs shows spherical microparticles with a uniform size distribution

406 (magnification ×2,000)

407 Right: A vial containing dried hMP powder

408 Abbreviations: hMPs, hydrophobized microparticles; SEM, scanning electron microscopy

409

410 **Figure 2. Endoscopic views of hMP application**

411 (a) Artificial gastric ulcer created via EMR-L

412 (b) Appearance immediately after spraying hMPs onto the ulcer base

413 (c) Formation of a hydrogel layer as hMPs rapidly gel upon contact with the moist ulcer surface

414 Abbreviations: hMPs, hydrophobized microparticles; EMR-L, endoscopic mucosal resection

415 with ligation

416

417 **Figure 3. Experimental design of the delayed bleeding model**

418 Artificial gastric ulcers are created using EMR-L (step 1), followed by the endoscopic spraying

419 of hMPs on the ulcer base (step 2). A bolus of heparin (50 U/kg) is administered until the ACT

420 exceeds 220 s (step 3). Continuous heparin infusion (50 U/kg/h) is initiated, and ACT is

421 monitored at 0.5, 1, 2, and 4 h (step 4). The animals are sacrificed 24 h after EMR-L, for

422 endoscopic and histological evaluations.

423 Abbreviations: hMPs, hydrophobized microparticles; EMR-L, endoscopic mucosal resection

424 with ligation; ACT, activated clotting time

425

426 **Figure 4. Time course of ACT following intravenous heparin administration**

427 Changes in ACT over time in three individual swine (numbers 1–3) are shown. After bolus

428 administration of heparin (50 U/kg), ACT is measured repeatedly, and continuous infusion (50

429 U/kg/h) is initiated when ACT exceeds 220 s. ACT values are subsequently monitored at

430 multiple time points during the infusion period.

431 Abbreviation: ACT, activated clotting time

432

433 **Figure 5. Endoscopic and gross anatomical images of the stomach 24 h after the procedure**

434 **in three swine**

435 Upper panels: Endoscopic views from swine numbers 1–3 showing ulcer bases covered with

436 hMPs

437 Lower panels: Gross anatomical views of resected stomachs showing no evidence of delayed

438 bleeding (no active bleeding or adherent clots)

439 Abbreviation: hMPs, hydrophobized microparticles

440

Figure 6. Ulcer base coverage by hMPs

Distribution of ulcer base coverage by hMPs in 36 ulcers. Complete coverage (100%) was achieved in 20 ulcers (55.6%), 75–99% coverage in 7 ulcers (19.4%), and 50–74% coverage in 9 ulcers (25.0%). None of the ulcers showed a coverage below 50%.

Abbreviation: hMPs, hydrophobized microparticles

Figure 7. Histological evaluation of ulcer bases after EMR-L and hMP application

The hMPs are retained on the ulcer base following EMR-L and are observed to cover the exposed blood vessels within the submucosa (a, c). Higher-magnification images (b, d) clearly show hMPs adhering to the surfaces of the exposed vessel-like structures.

Abbreviations: hMPs, hydrophobized microparticles; EMR-L, endoscopic mucosal resection with ligation

Figure 8. Proposed mechanism of delayed bleeding prevention by hMPs

Post-ESD ulcers are susceptible to damage and delayed bleeding under standard conditions. Spraying hMPs on the ulcer base creates a protective barrier, suppresses local inflammation, and prevents bleeding. hMPs can be easily delivered through an endoscope.

Abbreviations: hMPs, hydrophobized microparticles; ESD, endoscopic submucosal dissection

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