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23 inulin content and low gluten content. Solubilized inulin created a protective layer around
24 starch granules limiting their swelling and amylose release, which may explain the
25 differences in viscosity between the porridges and could have further influence in starch
26 digestibility.

27

28 **Highlights**

- 29 • Addition of inulin and gluten affected microstructure and texture of rye porridge
- 30 • FITC labeling allowed localizing solubilized inulin in the porridge
- 31 • Inulin accumulated preferentially around starch granules hindering their swelling
- 32 • Starch digestibility could be affected by the addition of inulin and gluten

33

34 **Keywords:** rye, starch, microscopy, fructan, viscosity

35 **1. Introduction**

36 Consumers are demanding healthier food products with improved functionalities and an
37 increasing number of new food formulations are being developed to satisfy this need. The
38 compatibility or incompatibility between ingredients in new food formulations can affect
39 both texture and structure (Icoz, Moraru, & Kokini, 2005). These structural changes could
40 also have later implications in the intended functionality of the product (McClements,
41 Decker, Park, & Weiss, 2009). When it comes to porridge, the rheological properties are of
42 great importance for quality control and consumer acceptance (Sai Manohar, Urmila Devi,
43 Bhattacharya, & Venkateswara Rao, 2011). Moreover, it can also have influence on the
44 satiating properties of the product (Mars, Hogenkamp, Gosses, Stafleu, & De Graaf, 2009).
45 Rye foods, which are important elements in the healthy Nordic diet, have shown favorable
46 effects on appetite (Isaksson et al., 2012), as well as beneficial effects on postprandial
47 insulin responses and inflammatory biomarkers (Fung et al., 2002; Landberg et al., 2010;
48 Rosén, Östman, & Björck, 2011). Addition of plant protein and fermentable dietary fiber
49 could possibly enhance the appetite suppressing effect of whole-grain rye porridge. Such
50 effects may in part be due to alterations in the microstructure of the product (Lundin,
51 Golding, & Wooster, 2008).

52 Inulin is an oligo-fructose polymer of interest in human nutrition due to its ability to act as
53 dietary fiber and prebiotic (Roberfroid, 2007). Due to its structure, inulin resists digestion in
54 the human intestine and is fermented by bacteria in the colon, which has been suggested to
55 affect appetite (Cani, Dewever, & Delzenne, 2004). Little work has been done to investigate
56 the effects of inulin on food structure. Microstructural studies of inulin-enriched products
57 have been carried out on cereal and dairy products (Aravind, Sissons, Fellows, Blazek, &
58 Gilbert, 2012; Guardedeño, Vázquez-Gutiérrez, Hernando, & Quiles, 2013; Guggisberg,

59 Cuthbert-Steven, Piccinali, Bütikofer, & Eberhard, 2009; Rodríguez-García, Puig, Salvador, &
60 Hernando, 2012; Sołowiej et al., 2015). However, the studies do not provide a detailed
61 localization of solubilized inulin in the structure and only insolubilized inulin crystals have
62 been detected by light microscopy (Guardeño et al., 2013). Interactions between inulin and
63 the protein structural network in yogurt have been suggested (Guggisberg et al., 2009; Kip,
64 Meyer, & Jellema, 2006), but such interactions have neither been properly described nor
65 confirmed by microstructural observations.

66 Gluten is found in the endosperm of cereals such as wheat, barley, and rye and is an
67 important by-product from wet milling of wheat flour. Wheat gluten is a common food
68 ingredient in bakery products such as hamburger buns (Esteller, Pitombo, & Lannes, 2005),
69 meat products as binding and enriching ingredient (Zhang, Xiao, Samaraweera, Lee, & Ahn,
70 2010), breakfast cereals, and pasta (Day, 2011). Wheat gluten is marketed in two forms:
71 'nonvital' and 'vital'. Nonvital wheat gluten has undergone irreversible denaturation, while
72 vital dry gluten in contact with water rehydrates rapidly and regains its intrinsic
73 functionality (Esteller et al., 2005). Therefore, vital gluten constitutes a desired additive in
74 baked and meat products due to its ability to form a viscoelastic mass through the
75 interaction with water (Esteller et al., 2005; Zhang et al., 2010). Interactions between gluten
76 and starch have been reported and supported by microscopy observation (Chen, Deng, Wu,
77 Tian, & Xie, 2010). It has also been suggested that there could be interactions between
78 gluten and inulin but this has not been confirmed by microstructural observation (Morris &
79 Morris, 2012; Peressini & Sensidoni, 2009; Rubel, Pérez, Manrique, & Genovese, 2015;
80 Wang, Rosell, & Bedito de Barber, 2002).

81 Labeling of inulin with fluorescein isothiocyanate (FITC) has been successfully used for
82 studies of the phase behavior of inulin-waxy maize starch systems (Zimeri & Kokini, 2003a).

83 To our knowledge, the method has so far only been used for model systems and this is the
84 first time that FITC labeling and localization of inulin by confocal microscopy is performed in
85 a complex food system. Previously only inulin crystals could be identified and the location of
86 soluble inulin could only be suggested in such systems, not proven by fluorescence signal as
87 in this study.

88 The aim of this study was to analyze the effect of partial substitution of rye flakes for inulin
89 and gluten on the microstructure and texture of whole grain rye flake porridge to obtain a
90 better understanding of the functionality of the product.

91

92 **2. Materials and Methods**

93 **2.1. Sample preparation**

94 Rye porridge was made from whole grain rye flakes, produced by steaming, cutting and
95 rolling rye kernels (Lantmännen Cerealia, Järna, Sweden). Four different samples were
96 prepared, one with 40 g rye flakes and the rest contained 40 g rye flakes with different
97 combinations of inulin (Orafti®GR inulin, purity 90%; Beneo, Mannheim, Germany) and
98 gluten (Vital Wheat Gluten, purity 77%; Arrowhead Mills, Boulder, USA). The combined
99 additions were recalculated to compensate for impurities to ensure ratios inulin/gluten of
100 1:3 (3 g inulin and 9 g gluten, 3I9G), 1:1 (6 g inulin and 6 g gluten, 6I6G) and 3:1 (9 g inulin
101 and 3 g gluten, 9I3G), as well as similar total weight of all the porridges. Samples were
102 prepared by adding boiling water (150 g) to the rye flakes/inulin/gluten mixtures and
103 manually stirred for 30 sec. The samples were then left to rest for 2 min and manually
104 stirred again for another 30 sec. The samples were left to rest for another 2 min, and then
105 deposited in aluminum caps and frozen with liquid nitrogen. Short-chain inulin (degree of
106 polymerization between 10 and 20) was chosen as it would have greater solubility than

107 long-chain inulin (Tárrega, Torres, & Costell, 2011) and would be expected to have less
108 effect on the viscosity of the product (Morris & Morris, 2012; Tárrega et al., 2011).

109

110 **2.2. Labeling method**

111 Inulin was covalently labeled with fluorescein isothiocyanate (FITC, Sigma-Aldrich Co. LLC.,
112 St Louis, MO) following the procedure described by Zimeri and Kokini (2003a) with
113 modifications. Briefly, inulin (1g) was dissolved in dimethyl sulfoxide (10 mL) containing two
114 drops of pyridine. FITC (0.04 g) was added, followed by addition of the catalyst dibutyltin
115 dilaurate (20 mg). The mixture was heated for 3 h at 50 °C using a water bath. Several
116 precipitations in ethanol were performed to remove the free dye. FITC-inulin was filtered
117 using a filter paper No. 3 (Whatman, Wand R Balston Ltd, England), dried overnight in a
118 vacuum oven at 85 °C, and stored in the dark under refrigeration to prevent loss of
119 fluorescence. In order to prepare the porridges, an amount of FITC-labeled inulin (1% of the
120 total inulin amounts described in section 2.1) was added before the mixing with hot water
121 and the sample preparation procedure outlined in section 2.1 was followed.

122

123 **2.3. Microscopy**

124 The frozen samples were transferred to a cryostat, and 8 µm cryosections were obtained
125 and placed in glass slides. Multiple staining was applied to cryosections, lugol's solution
126 (0.05 g/L iodine) to detect starch and protein (Groves, 2006), 0.1 g/L Calcofluor White for β-
127 glucan (Dornez et al., 2011), and 0.02 g/L Texas Red for protein (Johansson, Krona, &
128 Stading, 2012). A Nikon Eclipse Ni-U research microscope coupled to a HGFI mercury lamp
129 (Nikon, Tokyo, Japan) was used to visualize the microstructure of the porridges. Bright field
130 and epifluorescence images were obtained using CFI Plan 4X objective (N.A. 0.20, W.D. 20

131 mm) and CFI Plan Fluor 10X (N.A. 0.30, W.D. 16 mm) and 20X (N.A. 0.75, W.D. 1 mm)
132 objectives. Blue (Epi-FL Filterset DAPI, excitation wavelength 382-393 nm, emission 417-
133 477), green (Epi-FL Filterset FITC, excitation wavelength 465-500 nm, emission 516-556 nm),
134 and red (Epi-FL Filterset Texas Red, excitation 540-580 nm, emission 600-660 nm) light
135 fluorescence filters were used to observe the fluorescence of Calcofluor, FITC-inulin, and
136 Texas Red, respectively. Images were captured with a Nikon Digital Sight DS-Fi2-U3 digital
137 camera.

138

139 **2.4. Texture analysis**

140 A RVA (Rapid Visco Analyzer, Newport Scientific Pvt. Ltd., Australia) with an impeller-cup
141 combination was used to measure the viscosity of the porridges. Since rye porridge includes
142 particles in the millimeter range it is impossible to use rheometry with gap distances which
143 would give controlled shear rates and absolute measurements. For the RVA measurement
144 the average temperature, as measured with a thermocouple connected to a digital readout
145 during the preparation process described in section 2.1, was used. The rate profile was set
146 to simulate the stirring with an extra measurement period at the end of the run (Table 1).
147 For each different formulation, approximately 35 g of the sample were introduced in a
148 stainless steel cylinder and analyzed in the RVA in triplicate. The average viscosity during the
149 last 15 s of each measurement period was used to derive a viscosity profile for each
150 product. The first 15 s of the measurement periods were not included to avoid the initial
151 instabilities.

152

153

Table 1

154

155 **2.5. Statistical analysis**

156 Differences between viscosity profiles were evaluated using a mixed effect model suitable
157 for repeated measurements with PROC mixed in SAS, version 9.4 (SAS Institute Inc, Cary, NC,
158 USA). Time, product and a time x product interaction term were included as fixed effects
159 with time as a repeated variable. Sample was included as a random effect. When a
160 significant time x product interaction was found, Tukey's honest significance test was
161 performed for each time point.

162

163 **3. Results and discussion**

164 **3.1. Characterization of porridge structure by iodine staining**

165 After the addition of hot water to the whole grain rye flakes, starch granules swelled and
166 collapsed leading to gelatinization. The structure of the rye porridges, as visualized with
167 iodine staining, consisted of kernel fragments and an aqueous continuous phase with
168 released amylose (blue) and amylopectin (brown/purple), small fragments of starch
169 granules (dark blue/violet) and protein (yellow). Iodine staining, further allowed for
170 visualization of the aleurone and subaleurone cells, both rich in protein, as shown in yellow
171 color (Fig. 1A and B). Amylose and amylopectin seen in the continuous phase were released
172 from starch granules at the edge of the grain fragments as these had more access to water
173 and therefore underwent greater swelling. A few detached starch granules could be
174 distinguished in the aqueous phase, showing great level of distortion (Fig. 1C).

175 Starch granules in porridges containing inulin and gluten appeared to be less swollen than
176 the ones in the control product (Fig. 1D-F). This may be due to inulin and gluten competing
177 for water against the starch, owing to their water binding capacity, as has previously been
178 suggested in white sauces formulated with soy protein and inulin (Guardeño et al., 2013).

179 With no inulin and gluten added, more water was available for the starch, leading to greater
180 swelling of the granules.

181 The porridge with the highest amount of inulin (9I3G) showed smaller and less distorted
182 starch granules in the continuous phase compared with the sample with the highest amount
183 of gluten (3I9G). Moreover, the 9I3G presented less amount of released
184 amylose/amylopectin in the aqueous phase, which appeared less stained than in the other
185 samples.

186 According to Bishay (1998) and Manno et al. (2009), inulin has a greater affinity for the
187 water than starch polysaccharides and gluten. The water that is bound to inulin chains is
188 more mobile than when it is bound to the starch; this is simply due to inulin having shorter,
189 more mobile molecules (Lobato, Grossmann, & Benassi, 2009). The inulin's preferential
190 properties for hydrating, aggregating, and forming a matrix encase starch granules in a semi-
191 solid gel (Tolstoguzov, 2003). This encasing of the starch granules would possibly limit water
192 movement to the starch granules, reducing swelling and gelatinization (Brennan et al.,
193 2004).

194

195 Fig. 1

196

197 **3.2. Location of inulin by epifluorescence**

198 When samples were observed under epifluorescence, inulin labeled with FITC could be
199 detected as green fluorescence. The control sample, which did not contain inulin, presented
200 faint green autofluorescence from proteins and cell walls (Fig. 2A). However, strong green
201 autofluorescence was detected in the pericarp due to the high concentration of phenolic
202 acids in that area (Dornez et al., 2011). Therefore, the pericarp area appeared in light blue-

203 turquoise color in all the samples after combining blue and green fluorescence signals (Fig.
204 2A-D). Calcofluor staining allowed detecting β -glucan as blue fluorescence. High
205 concentration of β -glucan was observed in the aleurone and subaleurone layers, where
206 thick and relatively intact cell walls were observed. The blue fluorescence lost continuity and
207 became thinner towards deeper layers of the starch endosperm, indicating that cell walls
208 were damaged and β -glucan content was lower in those areas (Fig. 2A). Fragments of cell
209 walls with β -glucan could also be found in the aqueous phase among detached starch
210 granules and protein aggregates (Fig. 2B-D).

211 Green fluorescence signal from FITC-labeled inulin was observed heterogeneously
212 distributed in the aqueous phase and it was brighter as the amount of added inulin
213 increased. In the sample with lower amount of added inulin (3I9G), the green fluorescence
214 appeared to be slightly brighter in specific areas (Fig. 2B). These areas were stained in black-
215 dark blue color after lugol staining (Fig. 2F), corresponding to non-gelatinized starch
216 granules and aggregates of released amylose. In the samples with equal amount of added
217 inulin and gluten (6I6G), the fluorescence from the amylose aggregates became brighter
218 (Fig. 2C). Bright green fluorescence could also be observed around the gluten aggregates,
219 which were stained in yellow with lugol (Fig. 2G), which could indicate interactions between
220 inulin and gluten protein. The formulation with the highest amount of inulin (9I3G)
221 presented the brightest green fluorescence overall, especially located in the starchy areas
222 rich in amylose (Fig. 2D and H).

223 Some studies have not found evidence of interaction between inulin and amylopectin (Icoz
224 & Kokini, 2008; Zimeri & Kokini, 2003b). Therefore, it was concluded that the green
225 fluorescence observed in the starchy areas was due to interaction between inulin and
226 amylose or with starch granule-associated proteins.

227

228

Fig. 2

229

230 When samples were observed at higher magnification, bluish precipitates could be observed

231 around gluten aggregates after iodine staining in the porridges with medium and high

232 amount of inulin (6I6G and 9I3G), as shown in Fig. 3A. These precipitates could correspond

233 to released amylose, which would be interacting with the gluten protein. In the same way,

234 bright green fluorescence surrounding the gluten aggregates, which could be detected as

235 red fluorescence after Texas Red staining, could be observed (Fig. 3B). This indicated the

236 presence of inulin in similar areas where the amylose was located. Both amylose and inulin

237 could interact with gluten protein.

238

239

Fig. 3

240

241

242 **3.3. Texture analysis**

243 A statistically significant interaction between product x time was detected on the viscosity

244 profiles ($P < 0.05$). Therefore, Tukey's honest significance test was performed for each time-

245 point. All porridges exhibited increasing viscosity during the measurement (Figure 4). This

246 increase in viscosity was likely due to a series of changes taking place in the structure of the

247 porridges. The starch granules swell and amylose and amylopectin leach from the granules

248 resulting in increased viscosity. Simultaneously, granules rupture and amylose and

249 amylopectin chains align, contributing to decreased viscosity. Among the enriched

250 porridges, the final viscosity values significantly decreased ($P < 0.05$) with increasing inulin

251 content and decreasing gluten content (Figure 4). A similar trend was also seen at the two
252 first time points. Since the enriched porridges contained higher amount of dry matter
253 compared to the control porridge (more than 52 g for the enriched versus 40 g for the
254 control), all the enriched porridges would be expected to have higher viscosity values.
255 However, all enriched porridges, except the porridge with highest gluten content (3I9G), had
256 significantly lower ($P<0.05$) viscosity than the control one at all time points.

257

258 Fig. 4

259

260 The high water binding capacity of inulin and gluten decrease the water available for starch
261 gelatinization. Moreover, as observed in the microstructural study, inulin was located
262 preferentially around detached starch granules. The significantly lower ($P<0.05$) final
263 viscosity measured in the samples with higher inulin content (9I3G and 6I6G) could also be
264 due to the additional effect of inulin, encasing the starch granules, as observed in the
265 epifluorescence images (Fig. 2B-D). This would limit the release of amylose from the
266 granules and result in a smaller contribution from the starch network to the viscosity.
267 Gluten aggregates could also interfere with the starch network thereby decreasing the
268 viscosity. Chen et al. (2010) reported that type and amount of added gluten had
269 considerable influence on the mechanisms involved in the pasting properties of starch, such
270 as transportation of available water and transmission of gelatinization energy. However,
271 since the viscosity reached in the porridges decreased with increasing inulin content, it
272 seems that inulin had greater influence on the viscosity of the porridge than gluten (Figure
273 4). Moreover, as shown in the microstructural study, gluten occurs as bigger particles while
274 inulin seems to accumulate at surfaces with amylose. The higher content of particles in the

275 porridge with the highest gluten content could contribute to the increase in viscosity and
276 would explain the differences in viscosity observed between the porridges.

277 Unlike what has been previously reported in white sauces (Guardeño, Hernando, Llorca,
278 Hernández-Carrión, & Quiles, 2012; Guardeño et al., 2013), no signs of inulin insolubility or
279 recrystallization could be observed in porridges. Kim, Faqih, and Wang (2001) reported that
280 low concentrations of inulin such as 0.5 g/L do not lead to gel network formation after
281 heating at 80 °C. The concentration of inulin in the porridge with highest inulin content
282 (9I3G) was 0.67 g/L. According to Kim et al. (2001), approximately 2.5 g/L inulin can be
283 dissolved at 80 °C. Furthermore, short-chain inulin, as the one used in this study, is more
284 likely to remain solubilized than long-chain inulin (Tárrega et al., 2011). Therefore, it could
285 be concluded that inulin was completely dissolved in all the porridges and that
286 concentration was not sufficient for gel formation.

287 The protective effect of inulin on starch granules to reduce swelling and resist degradation
288 has previously been reported for inulin-enriched white sauces, leading to a less compact and
289 cohesive continuous phase (Guardeño et al., 2012; Guardeño et al., 2013). Since the
290 concentration of inulin reached in the porridges was relatively low, the density of inulin
291 chains would not be able to reach a critical crowding effect. Therefore, inulin would act as
292 diluent in the porridges without interacting synergistically with starch, as has been reported
293 for mashed potato (Alvarez, Fernández, Solas, & Canet, 2011). Contrarily, other studies on
294 inulin-enriched products, such as yoghurt, have reported a marked increase in the
295 consistency with the addition of inulin, which has been attributed to the generation of a
296 second network supporting the one of casein (Guggisberg et al., 2009). Kip et al. (2006)
297 concluded that inulin may also be partially involved in the formation of the protein
298 structural network during yoghurt fermentation by complexation with protein aggregates.

299 Differential scanning calorimetry analyses on potato starch-inulin gels have also suggested
300 that gelatinization of potato starch with inulin produced binary gel of common network that
301 could result from the competition of both components for water (Krystyjan, Ciesielski,
302 Khachatryan, Sikora, & Tomasik, 2015). Aravind et al. (2012) hypothesized that relatively
303 small amounts of inulin, similar to those added to rye porridge in this study, support
304 formation of a well-developed protein–fiber matrix subsequently acting as a physical barrier
305 to starch-degrading enzymes based on *in vitro* starch digestion tests. In this way, the
306 addition of inulin to rye porridge could limit starch digestibility and have later implications
307 for the glycemic response. Consequently it could potentially be utilized for the development
308 of products with reduced glycemic index.

309

310 **4. Conclusions**

311 Addition of inulin and gluten to rye porridge partly hindered starch gelatinization due to
312 their water binding capacity. Inulin was completely solubilized and preferentially located in
313 detached starch granules of the aqueous phase of the porridge, which could be due to
314 interaction between inulin and amylose molecules. The solubilized inulin would create a
315 protective layer around the starch granules limiting their swelling and the amylose release.
316 This protective matrix around the starch granules, together with the water binding capacity
317 of inulin and gluten, would explain the lower viscosity values observed in the porridges with
318 added inulin and gluten. On the other hand, the presence of gluten particles would
319 contribute to increased viscosity and could explain the higher viscosity observed for the
320 porridge with highest gluten content compared to the other enriched porridges. This feature
321 may lead to limited accessibility of starch-degrading enzymes, which could affect starch
322 digestibility *in vivo* and glycemic index.

323

324

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329

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433 **Figure captions**

434

435 **Fig. 1.** Light microscopy images of different porridge cryosections stained with lugol's solution. A-C)
436 Control; D) 3:9 inulin/gluten (3I9G); E) 6:6 inulin/gluten (6I6G); F) 9:3 inulin/gluten (9I3G). gf: grain
437 fragment; al: aleurone; ap: aqueous phase.

438

439 **Figure 2.** Microstructure of porridge with different inulin and gluten proportions: 0:0 (control), 3:9
440 (3I9G), 6:6 (6I6G), 9:3 (9I3G). Epifluorescence images (top row) with Calcofluor White staining (blue
441 fluorescence for β -glucan and green fluorescence for FITC-labeled inulin) and bright field images
442 (bottom row) with iodine staining (protein in yellow, amylose in blue, amylopectin in purple). Red
443 arrows: inulin rich areas (top row) colocalized with starch areas (bottom row). Same areas are shown
444 in both rows.

445

446 **Fig. 3.** Microstructure of rye porridge with equal amounts of added gluten and inulin (6I6G). A)
447 Bright field with iodine staining; B) Epifluorescence with Calcofluor and Texas Red staining. Black
448 arrows: amylose precipitates; white arrows: inulin; gl: gluten aggregate.

449

450 **Fig. 4.** Viscosity profiles of rye porridge with different inulin and gluten proportions derived from
451 measurement periods in the RVA. Control (—■—), 3:9 (···◆···), 6:6 (—▲—), 9:3 (—●—). Values are least
452 square means \pm standard errors. Different letters at specific time points indicate statistically
453 significant differences between products ($P < 0.05$).

Table 1. Conditions for the RVA test on the porridge samples (total duration 330 s)

Step	1	2	3	4	5
Temperature (°C)	75	75	75	75	75
Duration (s)	30	120	30	120	30
Agitation (rpm)	30	0	30	0	30

Figure 1
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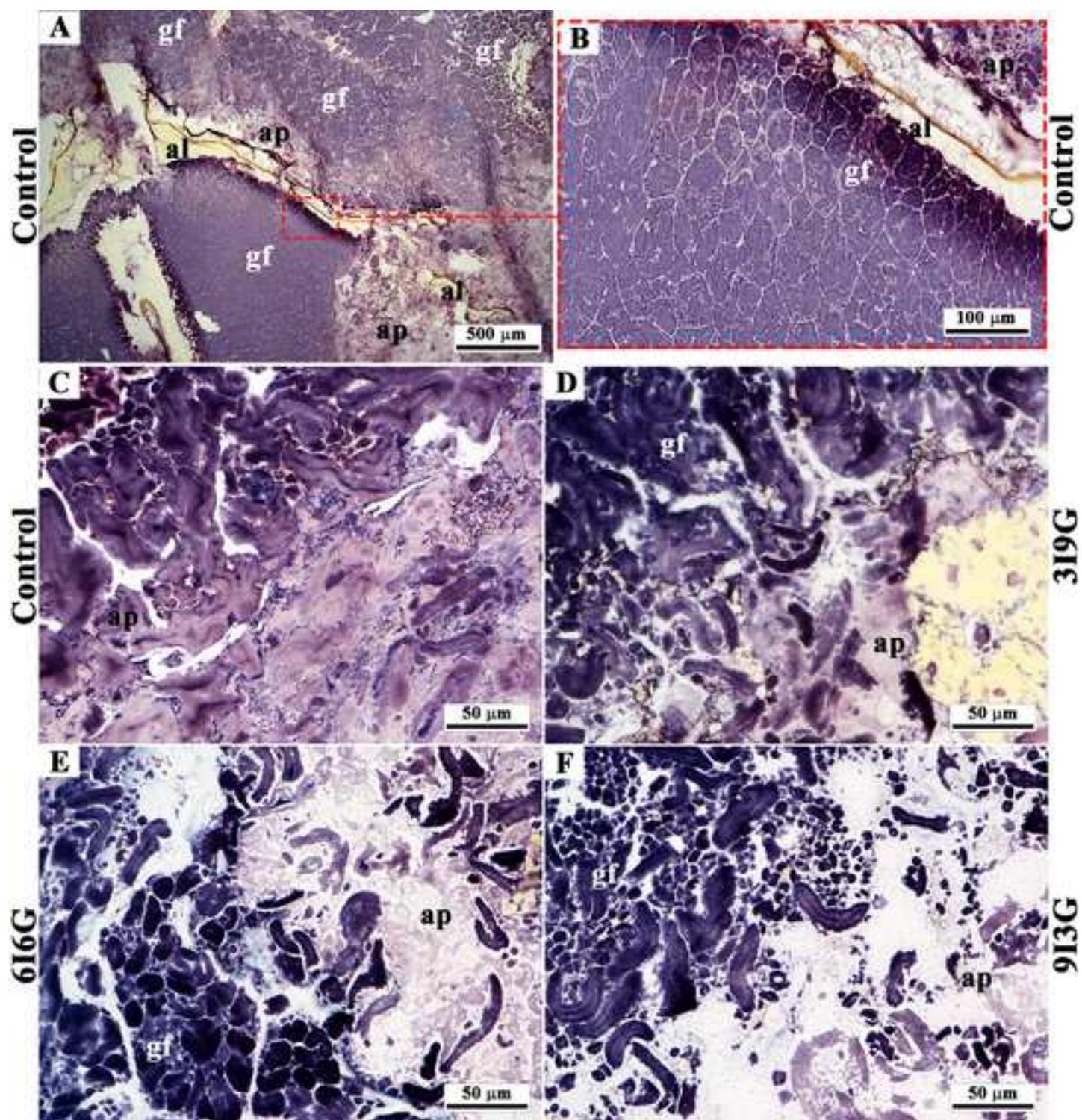


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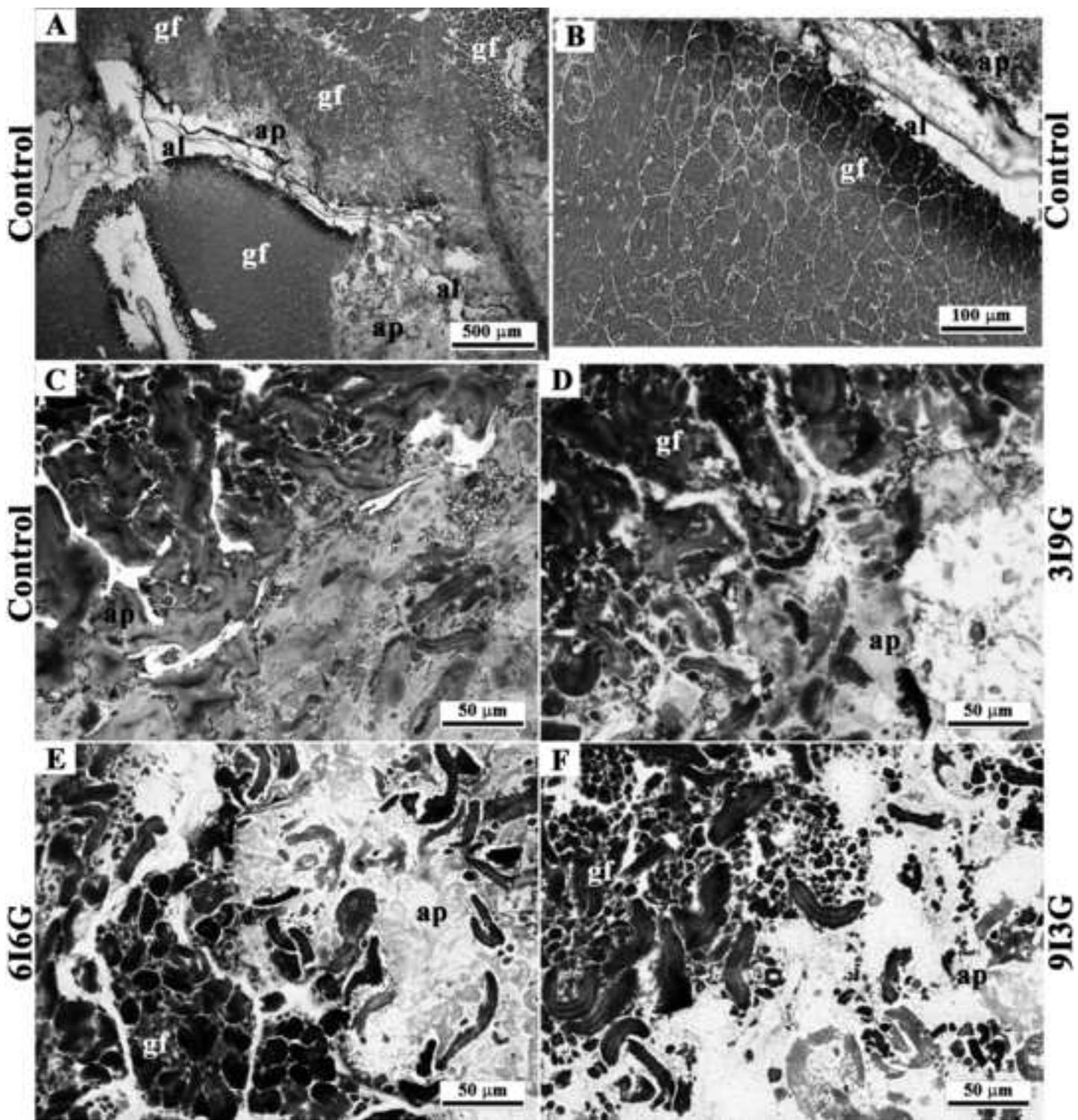


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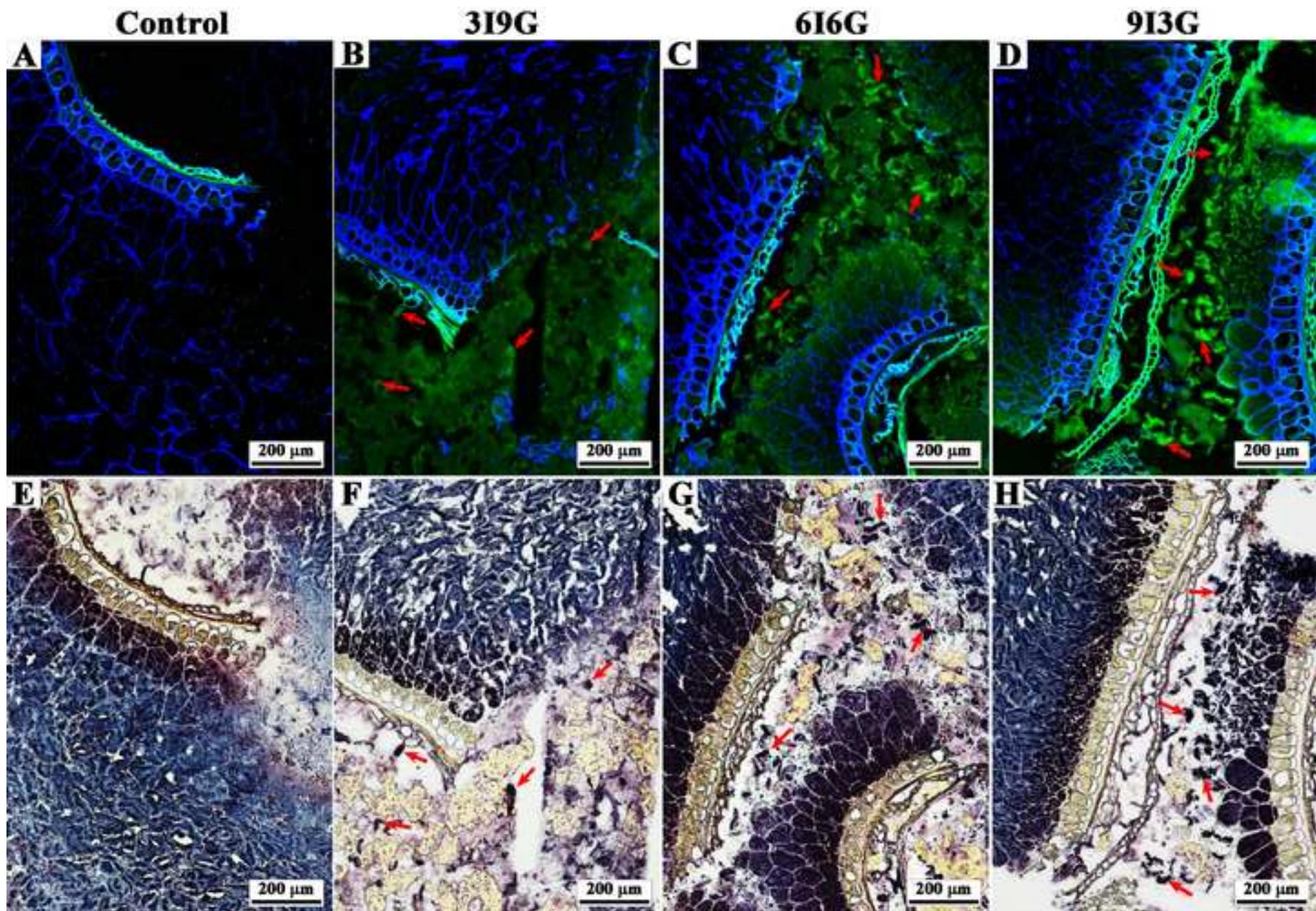


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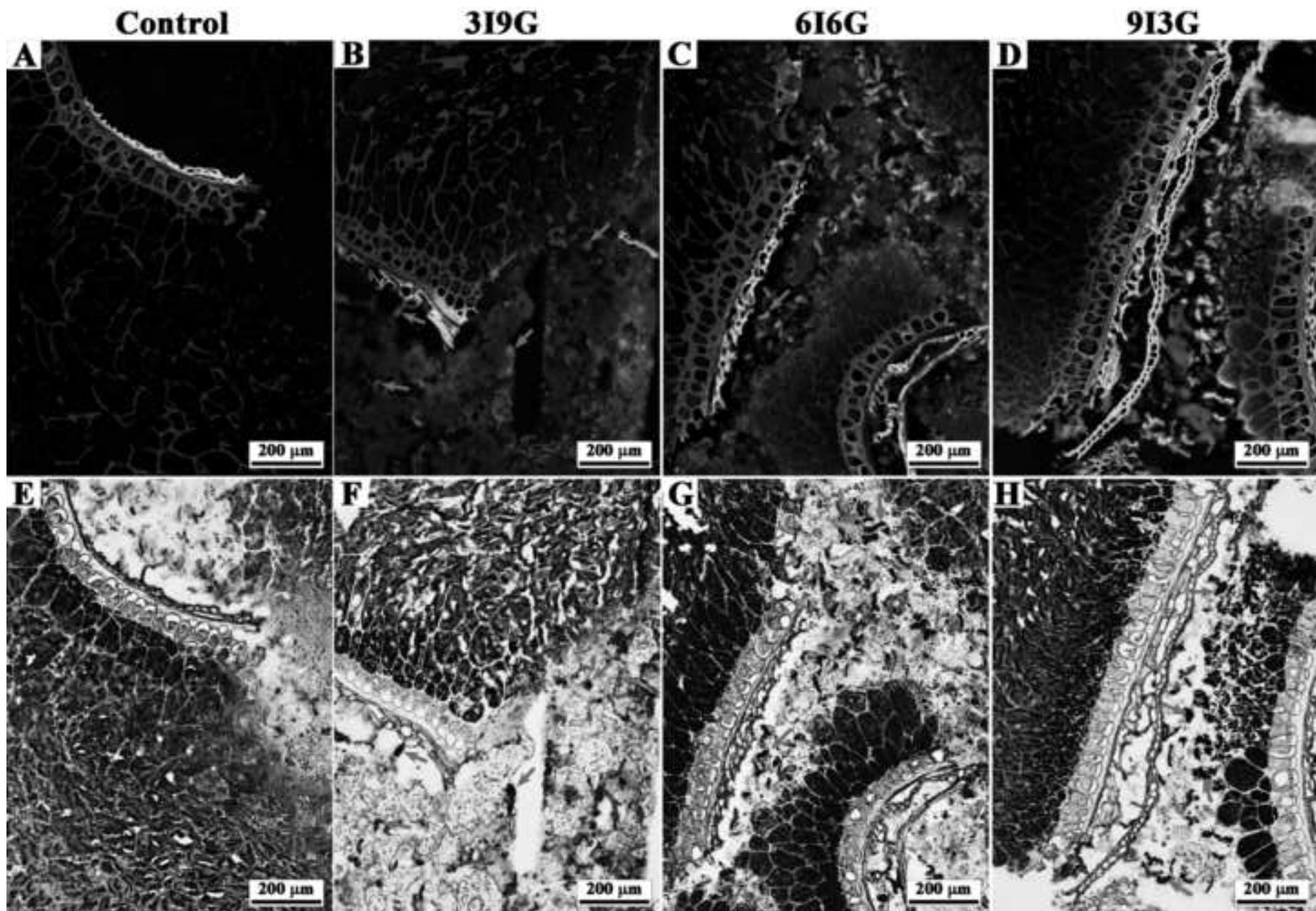


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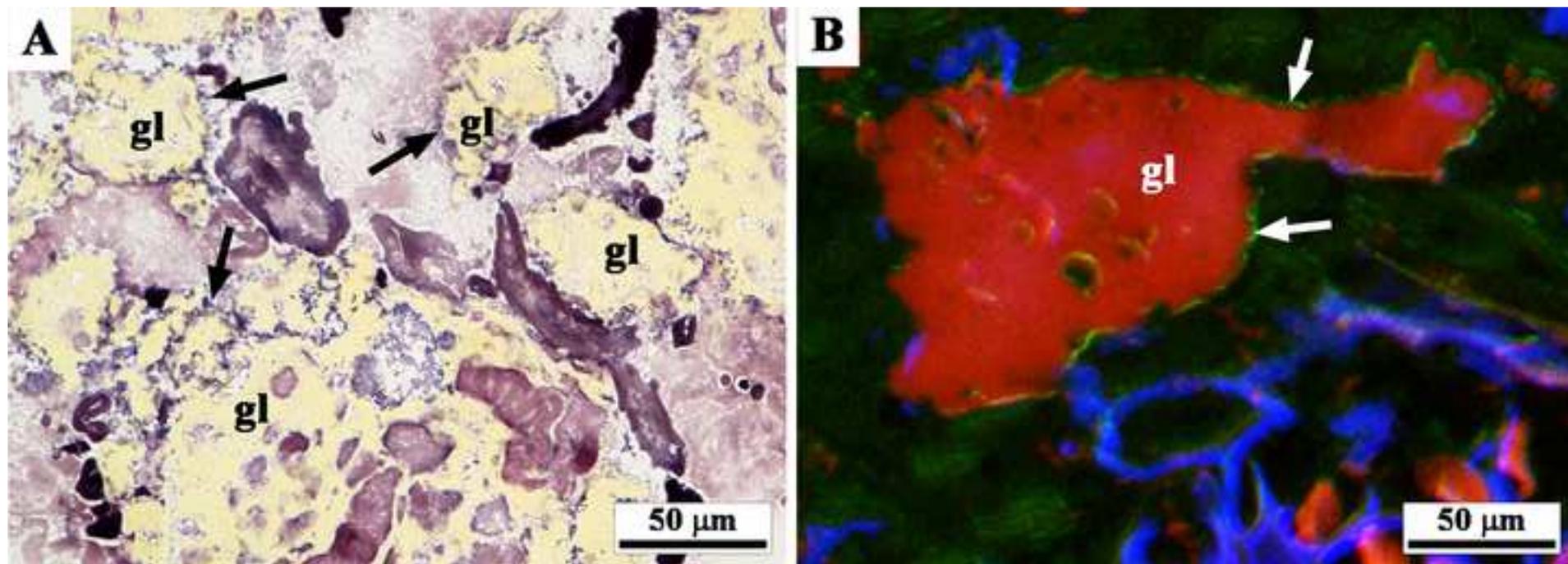


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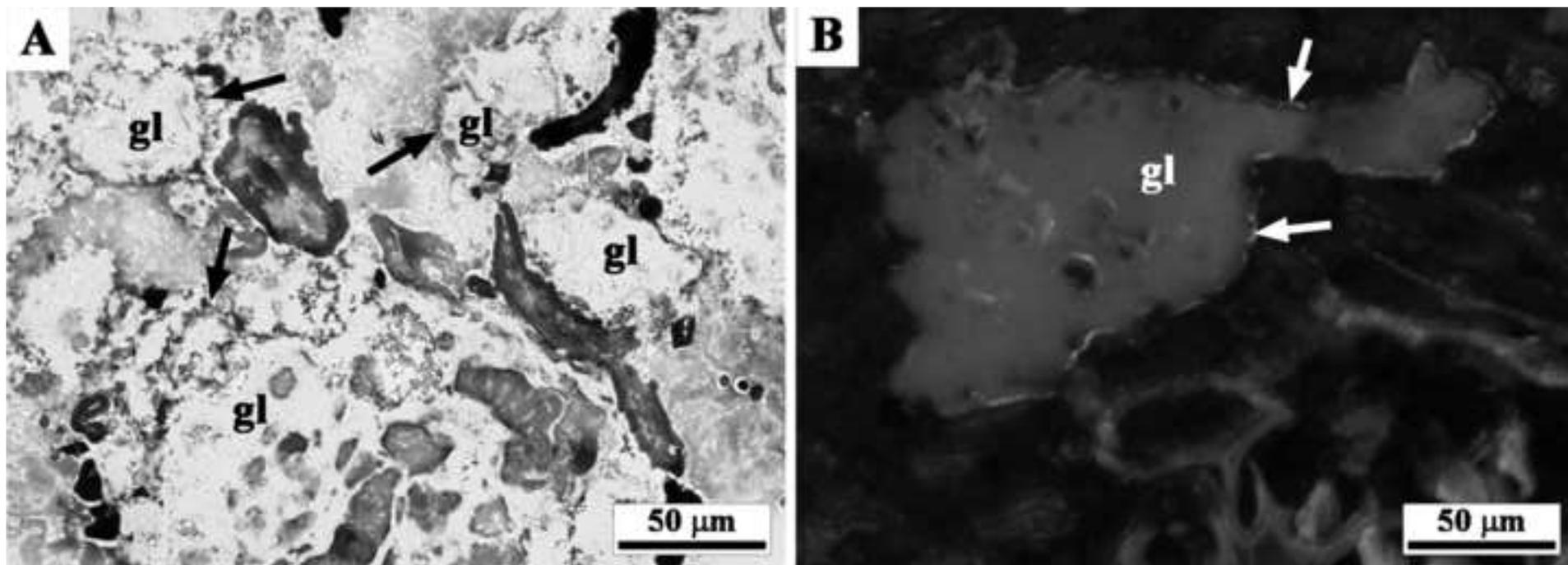


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