Promotion of osteogenesis and angiogenesis in vascularized tissue-engineered bone using osteogenic matrix cell sheets

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Abstract

Background: The regeneration of large, poorly vascularized bone defects remains a significant challenge. Although vascularized bone grafts promote osteogenesis, the required tissue harvesting causes problematic donor site morbidity. Artificial bone substitutes are promising alternatives for regenerative medicine applications, but the incorporation of suitable cells and/or growth factors is necessary for their successful clinical application. The inclusion of vascular bundles can further enhance the bone forming capability of bone substitutes by promoting tissue neovascularization. Little is known about how neovascularization occurs and how new bone extends within vascularized TEB (VTEB), because no previous studies have used tissue-engineered bone (TEB) to treat large, poorly vascularized defects.

Methods: In this study, we developed a novel VTEB scaffold composed of osteogenic matrix cell sheets wrapped around vascular bundles within β-tricalciumphosphate ceramics.

Results: Four weeks after subcutaneous transplantation in rats, making use of the femoral vascular bundle, VTEBs demonstrated more angiogenesis and higher osteogenic potential than the controls. After VTEB implantation, abundant vascularization and new bone formation were observed radially from the vascular bundle with increased mRNA expression of alkaline phosphatase, bone morphogenetic protein-2, osteocalcin, and vascular endothelial growth factor-A.

Conclusion: This novel method for preparing VTEB scaffolds may promote the regeneration of large bone defects, particularly where vascularization has been compromised.

Background

Vascularized bone grafting is widely used to treat massive bone defects following trauma or tumor resection, osteomyelitis, or osteonecrosis, and is also useful for difficult situations in hand surgery. Although vascularized bones contain high osteogenic potential, the graft procedure is associated with difficult harvesting and complications at donor sites [1, 2].

Artificial bone material has gained attention as an implant that differs from metallic and polymeric materials in its superior compatibility with *in vivo* bone; therefore, it has a broad range of clinical applications [3, 4]. Although the material possesses some osteoinductive and osteoconductive activity, the osteogenic potential of artificial bone is limited [5]. Factors enhancing neovascularization are required for new bone formation in artificial bone materials to promote osteogenic differentiation and proliferation.

Previous research has explored methods to enhance neovascularization within artificial bone. Tissue-engineered techniques, in which bone marrow stromal cells (BMSCs) or growth factors are added to artificial bone [6–9], have enabled new procedures for bone regeneration. Although there have been several clinical trials using tissue-engineered bone (TEB) grafts [10, 11], we know of no previous studies treating patients with massive bone defects using TEB grafts, particularly in poorly vascularized areas occurring post irradiation or infection-derived scarring lesions.

In a previous study, bone regeneration was promoted by inserting vascular bundles into tissue-engineered madreporic coral implants [12], and several subsequent studies have been performed to expedite vascularization of TEB grafts [13–17]. These studies included methods for promoting neovascularization by inserting vascular bundles with mesenchymal stem cells (MSCs) and/or growth factors into the artificial bone. This combination is critical for successful bone regeneration in difficult clinical situations with massive bone defects and devascularized surrounding structures [18, 19]. However, there is little information on how

50 neovascularization occurs and newly formed bone extends within the vascularized TEB
51 (VTEB). In addition, there are no previous studies comparing different tissue engineering
52 techniques by evaluating the growth factors that promote angiogenesis and osteogenesis.
53 We developed a novel cell transplantation technique for bone formation using rat osteogenic
54 matrix cell sheets (OMCS) [20]. Because OMCS do not require a scaffold and maintain
55 intercellular networks with the extracellular matrix that they produce, these sheets can be
56 used in various graft sites in animal models. Because OMCS actually produce growth factors,
57 such as bone morphogenetic protein and vascular endothelial growth factor, they are an ideal
58 candidate for simultaneously promoting new bone formation and neovascularization. The
59 combination of OMCS and vascular bundles in the center of artificial bone could quickly
60 induce neovascularization and create a three-dimensional vascular network within the
61 artificial bone. This could encourage early new bone formation within the artificial bone and
62 allow early integration with the surrounding bone tissue.
63 We hypothesized that using OMCS with vascular bundles could enhance angiogenesis and
64 osteogenesis of β-tricalciumphosphate (β-TCP), enabling the generation of a VTEB scaffold
65 with osteogenic potential. To prove this hypothesis, the study was designed (1) to prepare a
66 VTEB by inserting a vascular bundle wrapped with OMCS into scaffold constructs, i.e.,
67 VTEB is fabricated by OMCS; (2) to histologically observe new bone formation and
68 neovascularization of this VTEB; (3) to quantitatively evaluate angiogenesis and osteogenesis
69 of VTEB; and (4) to compare this VTEB and a vascularized BMSC-mediated β-TCP for
70 angiogenic and osteogenic potential.

Methods

Bone marrow cell preparation

Bone marrow cells were prepared as previously described [21, 22]. In brief, bone marrow cells were obtained from the femoral shafts of 7-week-old male Fischer 344 rats by flushing with 10 ml of culture medium. The released cells were collected in two 75-cm^2 culture flasks (Falcon, BD Biosciences, Franklin Lakes, NJ, USA) containing 15 ml of regular medium comprising minimal essential medium (MEM, Nacalai Tesque, Kyoto, Japan) supplemented with 15% fetal bovine serum (FBS; Gibco Life Technologies, Carlsbad, CA, USA) and antibiotics (100-U/ml penicillin and 100-μg/ml streptomycin; Nacalai Tesque, Kyoto, Japan). Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. After reaching confluence (approximately day 14), the primary cultured cells were released from the culture substratum using trypsin–ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque Inc, Kyoto, Japan).

Osteogenic matrix cell sheet preparation and cell culture

After the primary culture, to create osteogenic matrix sheets, the released cells were seeded at 1×10^4 cells/cm^2 onto 10-cm dishes (5.8×10^5 cells/dish) for subculture in regular medium containing 10 nM of dexamethasone (Sigma, St. Louis, MO, USA) and 82-μg/ml L-ascorbic acid phosphate magnesium salt n-hydrate (Wako Pure Chemical Industrials, Kyoto, Japan) until they reached confluence (approximately day 14). After two rinses with phosphate-buffered saline (PBS; Gibco, Life Technologies, Carlsbad, CA, USA), the cell sheet was lifted using a scraper. The cell sheet was easily detachable from the culture dish by gentle scraping in PBS, starting from the periphery of the sheet (Figure 1a).

Cell viability assay

To investigate the viability of OMCSs after 2 weeks sub-culture prior to implantation into the β-TCP scaffold, a method based on tetrazolium reductase activity (Cell Counting Kit-8®; WST-8, Dojindo, Kumamoto, Japan) was employed [23]. Briefly, OMCSs cultured in 6-cm dishes and 12- and 24-well plates (Falcon, BD Biosciences, Franklin Lakes, NJ, USA; n = 5) were used to generate a standard. The differently sized OMCSs were harvested with a scraper, and then incubated in a 95% humidified atmosphere with 5% CO^2 at 37 °C for 24 h. The samples were then placed in WST-8 solution (100 μl in 1 ml of culture medium) in culture wells. After 3 h of incubation, the solution obtained from each culture well was analysed using a spectrophotometer (450 nm). Based on the standardization, a linear relationship was obtained between the averaged optical density and seeded cell number per volume of culture medium (cell/ml) (correlation $R^2 = 0.9983$). For all specimens, the cell viability of OMCSs after harvest with a scraper was calculated as a percentage compared with the cell viability of OMCSs before harvesting.

BMSC transfer onto β-TCP

The cylindrical β-TCP ceramic scaffold was prepared by HOYA Corporation (Tokyo, Japan). The cylindrical scaffold (diameter, 6 mm; length, 10 mm) had a side groove (width, 2 mm) connecting the center of the scaffold, which passed through the scaffold along its long axis (Figure 1b). This scaffold was highly porous with fully interconnected pores (porosity, 75% ± 3%; spherical pores, 200 ± 100 μm in diameter; interconnected channel, 75 ± 25 μm in diameter; micropores, 0.5–10 μm). The pores were well interconnected and opened into the central tunnel and outer surface of the scaffold. After release with trypsin–EDTA, BMSCs were centrifuged at 900 rpm for 5 min at room temperature, and resuspended to 3.5×10^6 cells/ml in MEM. BMSCs were counted using a hemocytometer and loaded onto the β-TCP scaffold (n = 8; 5.8×10^5 BMSCs/scaffold). Each scaffold was transferred into 12-well plates

(Falcon, Franklin Lakes, NJ, USA) for subcultures (Figure 1c); they were subcultured in one well with 2.5 ml of the regular medium containing 10 nM dexamethasone and 82 μg/ml L-ascorbic acid phosphate magnesium salt n-hydrate. The medium was renewed three times a week, and the subcultures were maintained for 2 weeks. After 2 weeks of subculture, each scaffold was implanted in syngeneic rats.

Animal care and handling

Our institute's Animal Care Committee approved the care and handling of the rats used in this study, which met the standards of the National Institutes of Health.

Surgical procedures and experimental groups

Syngeneic 11-week-old Fischer 344 rats were anesthetized by intramuscular injection of pentobarbital (3.5 mg per 100 g of body weight) after light ether inhalation. Both sides of the femoral vascular bundle were exposed under a microscope, and the vascular bundle was passed through the groove of the β-TCP. We designed the following three groups (Group V, Group cV and Group sV, n = 8 in each group) (Figure 2a and 2b). Samples from each group were extirpated 4 weeks after implantation to compare the histological images and the results of angiogenesis and osteogenesis by real-time quantitative polymerase chain reaction (PCR).

Histological analysis

Implants from each group (4 weeks after implantation) were fixed in 10% buffered formalin and decalcified using 10% EDTA/PBS. Sections were then cut and stained with hematoxylin and eosin (H&E), osteocalcin and CD31 for light microscopic observation.

Biochemical analysis

We conducted real-time quantitative PCR (TAQMAN, Life Technologies, Carlsbad, CA, USA) to measure mRNA expression levels of alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP-2), osteocalcin (OC), and vascular endothelial growth factor-A (VEGF-A) using primers and specific fluorogenic probes. Target mRNA levels were compared after correcting to glyceraldehyde-3-phosphate dehydrogenase mRNA levels as an internal standard, which was used to adjust the differences in the efficiency of reverse transcription between samples.

Statistical analysis

The values for real-time quantitative PCR are represented as means and standard deviations. Statistical significance was determined by one way analysis of variance post-hoc multiple comparisons using Tukey's test, and values of $p < 0.05$ were considered statistically significant.

159 ## Results

160 ### Viability of OMCSs

161 Compared with the cell viability of OMCSs before harvesting, the cell viability of OMCSs
162 prior to implantation was 48.8 ± 1.3%.

163

164 ### Histological analysis

165 The results of the histological images 4 weeks post-implantation (H&E stain) are shown in
166 Figures 3a–3f. In addition, Figures 4ab and 4cd show osteocarcin and CD31
167 immunohistochemistry results of Group sV, respectively. There were no obvious signs of
168 inflammation observed in any of the groups. In group V, there was no neonatal bone inside
169 the β-TCP, whereas there was limited neovascularization at the outer edge and inside the β-
170 TCP. In group cV, although the neonatal bone was localized to the outer edge of β-TCP and
171 slight neovascularization was present at the outer edge and inside the β-TCP, there were no
172 signs of robust vascularization or new bone formation around the vascular bundle in the
173 centre of the β-TCP. In group sV, there was extensive vascularization and new bone
174 formation radially from the vascular bundle. In addition, there was a limited amount of
175 neonatal bone and neovascularization in the outer edge of the construct.

176

177 ### Biochemical analysis

178 The expression of mRNAs in the constructs at 4 weeks after implantation was evaluated by
179 real-time quantitative PCR. The mRNAs levels of ALP, BMP2, OC, and VEGF-A were
180 significantly higher in the group sV than that in the other groups ($p < 0.001$). The mRNA
181 levels of ALP and OC were higher in group cV than in group V ($p < 0.01$), whereas no
182 significant difference was observed between the levels of BMP2 and VEGF-A in either group
183 V or cV (Figure 5).

Discussion

Vascularization strategies have gained interest in the field of tissue engineering because a well-vascularized environment is a prerequisite for successful cell engraftment and organ-specific function of the transplanted cells. Pelissier et al. [12] investigated vascular and bone ingrowth ratios in MSC TEB and compared the ratio between groups with and without vascular bundle implantation. The authors described that vascular bundle implantation in the central area of the implants significantly increased vascularization throughout the grafts, whereas vascular infiltration from peripheral muscular tissue failed to reach the central region in the absence of vascular bundles. Kawamura et al. [14] established an experimental model that showed the consistent potential of bone formation of vascularized MSC/hydroxyapatite implants by transferring them into lesions lacking adequate blood supply. Wang et al. [15] demonstrated higher bone ingrowth when a vascular bundle was implanted along with autologous bone marrow MSCs plus β-TCP in a rabbit femur segmental defect model. These studies suggested that the insertion of vascular bundles into various kinds of TEB is essential to promote bone growth in the artificial bone construct, particularly in circumstances with poor vascularity.

Both of the cell-derived techniques used in our study, including OMCS and BMSC suspension, successfully regenerated new bone formation in the β-TCP construct compared with the materials with vascular bundle implantation alone. On the basis of our gross histological observations, the inserted vascular bundles radially sprouted a capillary vessel network in the pores of the β-TCP scaffold, which was dramatically enhanced by wrapping the bundle in OMCS. Despite the lack of cell suspension in the scaffold, this OMCS wrapping technique promoted increased neovascularization, follow-up mineralization, and new bone regeneration. This is evidenced by the fact that the majority of the new bone was formed in the central region near the inserted vessels. Moreover, new bone formation in the

OMCS group was higher than that in the BMSC suspension group. The latter group demonstrated fibrous tissue interposition between β-TCP and the vascular bundle. We compared angiogenesis and osteogenesis using different cell-derived techniques of TEB.

Cell sheet engineering was developed as an alternative approach to improve BMSC-mediated tissue regeneration and was designed to avoid the shortcomings of traditional tissue engineering. The use of continuous cell sheets may be beneficial for cell transplantation, particularly in bone tissue engineering applications, because of the preservation of cellular junctions, endogenous extracellular matrix, and mimicry of the cellular microenvironment. The current mechanical retrieval method using a cell scraper creates OMCS, demonstrating their potential to form bone tissue without the necessity of a scaffold [20, 24]. Although OMCS have been demonstrated to retain their osteogenic potential [25], they have not been used for the production of TEB with a vascular bundle. We demonstrated the effectiveness of OMCS compared with BMSC suspension for new bone formation inside an artificial bone.

The samples in the OMCS group expressed significantly higher levels of ALP, OC, and BMP2 mRNA compared with the BMSC suspension group at 4 weeks after implantation. This result indicates that OMCS enhanced osteogenesis in the vascular bundle-implanted β-TCP to a greater degree. Additionally, the samples in the OMCS group expressed higher levels of VEGF mRNA. VEGF is a vital angiogenic factor that is predominantly produced in tissues that acquire new capillary networks [26, 27]. VEGF also contributes to the upregulation of BMP-2 in endothelial cells, indicating the interactive relationship of the signaling pathways between endothelial and osteoblastic lineage cells [28]. The significant increase in mRNA expressions of ALP, OC, and BMP2 can be explained by the fact that OMCS contains extracellular matrix, which is responsible for transmitting chemical and mechanical signals that mediate key aspects of cellular physiology [29]. The storage and

233 release of various growth factors by the extracellular matrix, which is produced by OMCS,
234 may have offered distinct advantages for angiogenesis and osteogenesis in our experiment.
235 There are several limitations to our study. First, the selection and characteristics of the
236 vascular bundle may influence the angiogenic effect on TEB. There are several donor vessels
237 for prefabrication of axially vascularized bone flaps in different animal models [30-33]. In
238 our study, a flow-through femoral vascular bundle was chosen, essentially running in a side-
239 by-side fashion without significant arteriovenous communication [34]. However, the femoral
240 vessel is thick and easy to treat, leading to a minimum possibility of vascular obstruction [12].
241 Second, the extrapolation of BMSCs from small animal models carries the risk of
242 unpredictable overestimation of the vascularization properties because neither the matrix
243 geometry nor the tissue-matrix-loop interactions are comparable. Experiments in larger
244 animals to increase the size of the model are the subject of future studies. Finally, harvesting
245 reduced the cell viability of OMCSs to approximately half. However, we confirmed that
246 implantation of these OMCSs led to high levels of osteogenesis and angiogenesis. We
247 suppose that the preparation of a highly viable cell sheet using a thermoresponsive culture
248 dish [35] further enhances osteogenesis and angiogenesis.
249

250 ## Conclusions

251 In this study, we demonstrated that using OMCS with a vascular bundle in the center of a β-
252 TCP scaffold maintained a high angiogenic and osteogenic potential 4 weeks after
253 implantation and efficiently enhanced new bone formation within the β-TCP scaffold. This
254 method using OMCS is expected to be a powerful tool for preparation of VTEB with high
255 angiogenesis and osteogenesis.

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Figure legends

Figure 1

1a: Macroscopic appearance of an osteogenic matrix sheet. Bone marrow stromal cells (BMSCs) cultured with dexamethasone and vitamin C were lifted as a cell sheet structure using a scraper;

1b: Microporous structures of the cylindrical β-tricalciumphosphate (β-TCP) scaffold with a side groove;

1c: The BMSC/scaffold construct was subcultured; and

Figure 2

2a: Group V, the vascular bundle was implanted into the β-TCP; Group cV, the vascular bundle was implanted into the β-TCP in which the BMSC suspension was performed; and Group sV, the vascular bundle surrounded by OMCS was implanted into the β-TCP.

2b: The femoral vascular bundle was inserted into the side groove with the osteogenic matrix cell sheets (OMCS).

Figure 3

Histological observation at 4 weeks after implantation (hematoxylin and eosin stain).

In group sV (Figure 3ef), new bone formation and vascularization was greater than that in group cV (Figure 3cd), whereas group V (Figure 3ab) showed no neonatal bone although a small amount of vascularization was observed in β-tricalciumphosphate (β-TCP).

Figure4

Immunohistochemistry using osteocalcin (ab) and CD31 (cd) in Group sV. New bone formation and neovascularization inside β-TCP are visible.

381

382 Figure 5

383 The mRNA expression levels in each group at 4 weeks after implantation [data shown as
384 mean ± standard deviation (SD), n = 6]. (*, $p < 0.001$; †, $p < 0.01$).

385 ALP: alkaline phosphatase; BMP2: bone morphogenetic protein 2; OC: osteocalcin, VEGF-
386 A: vascular endothelial growth factor-A; and GAPDH: glyceraldehyde-3-phosphate
387 dehydrogenase.

Figure1A
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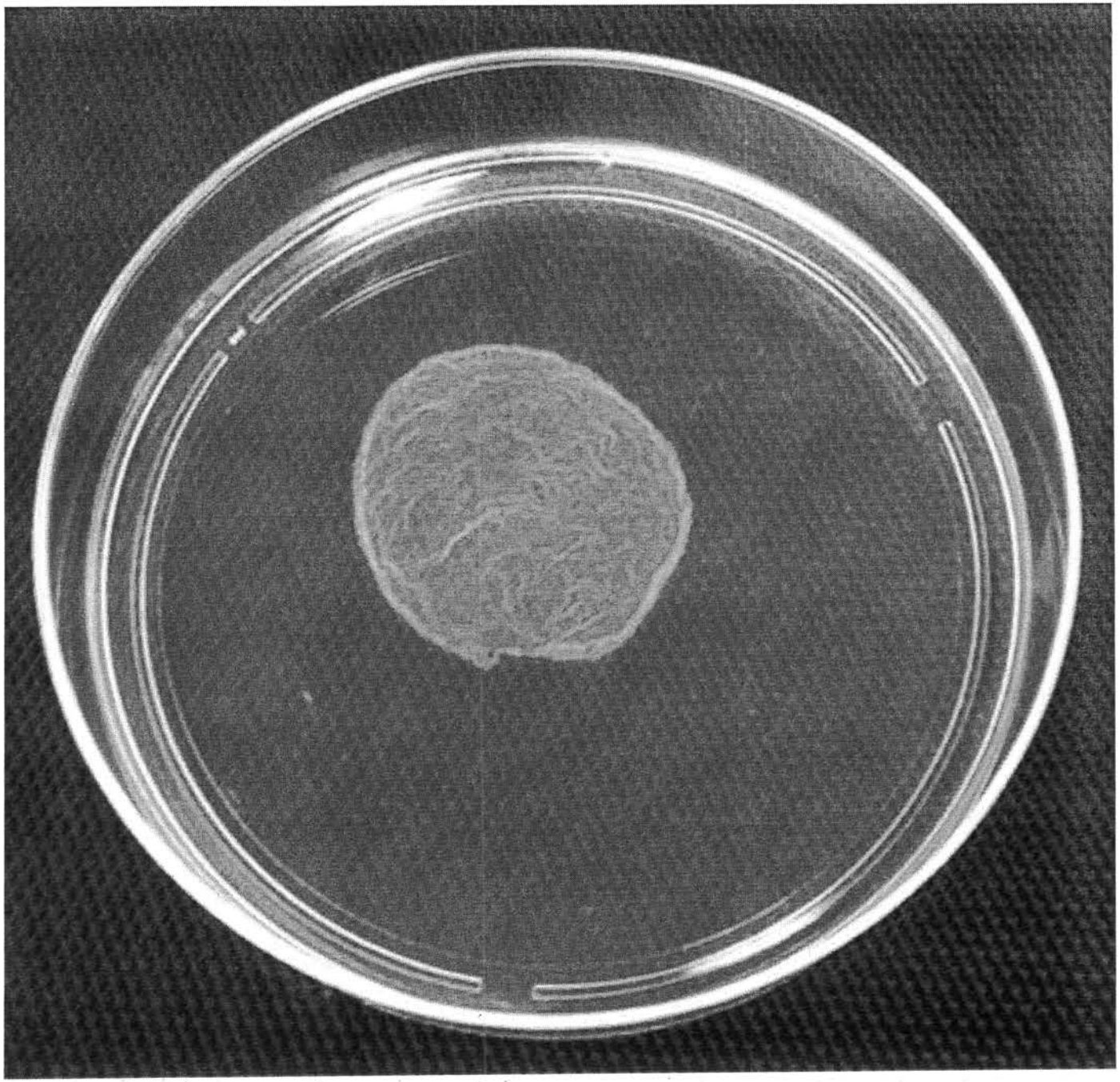

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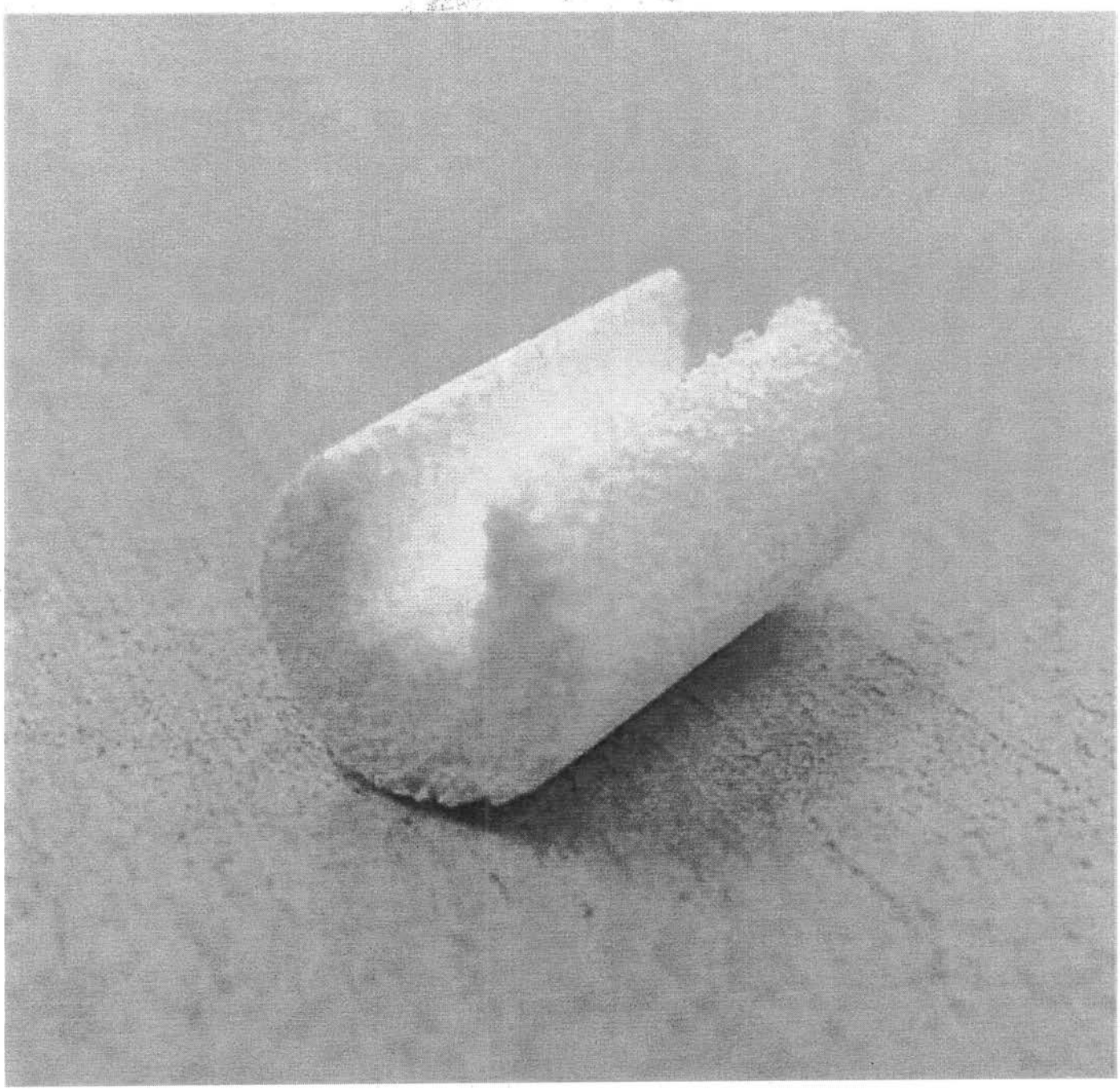

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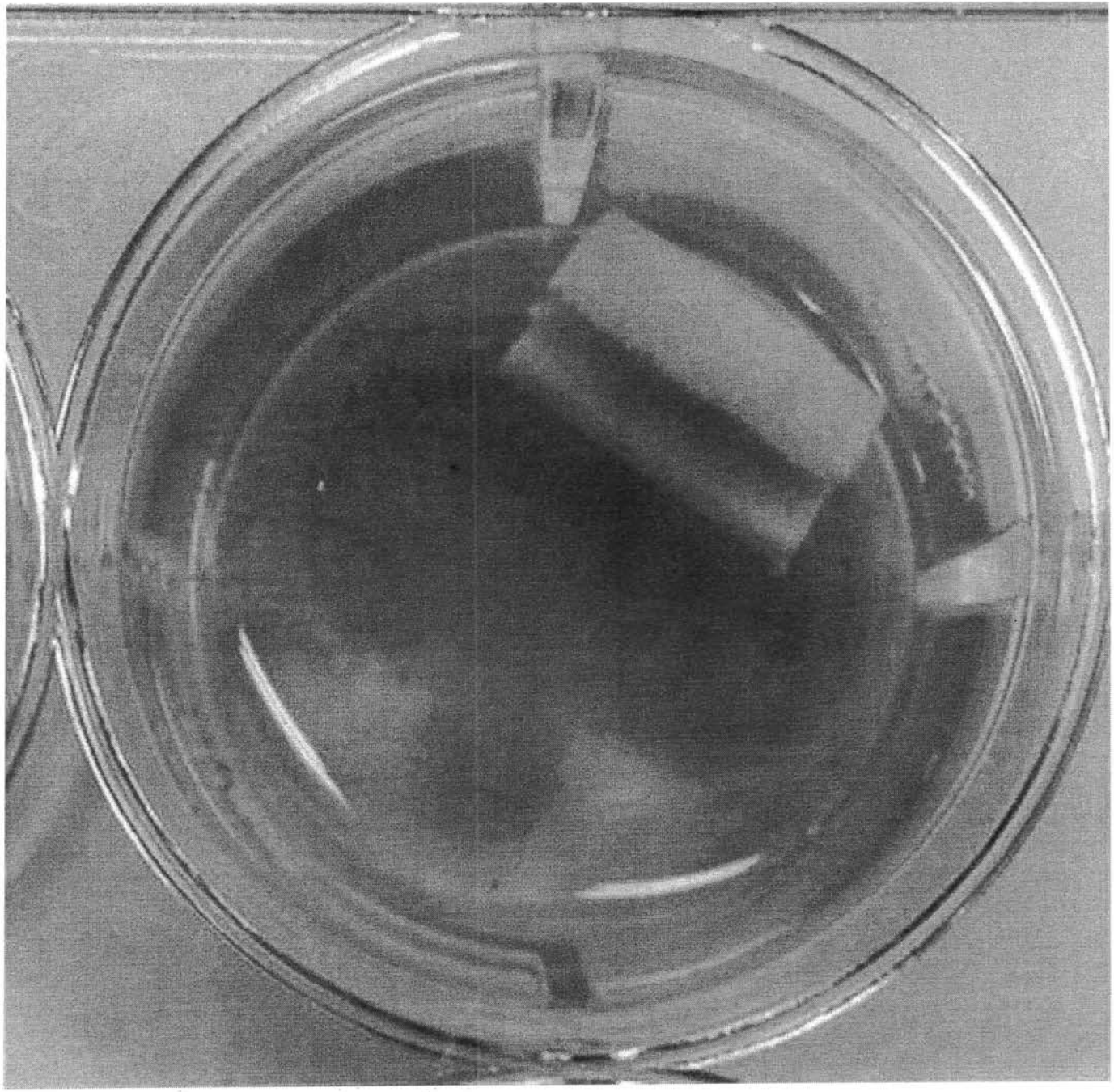

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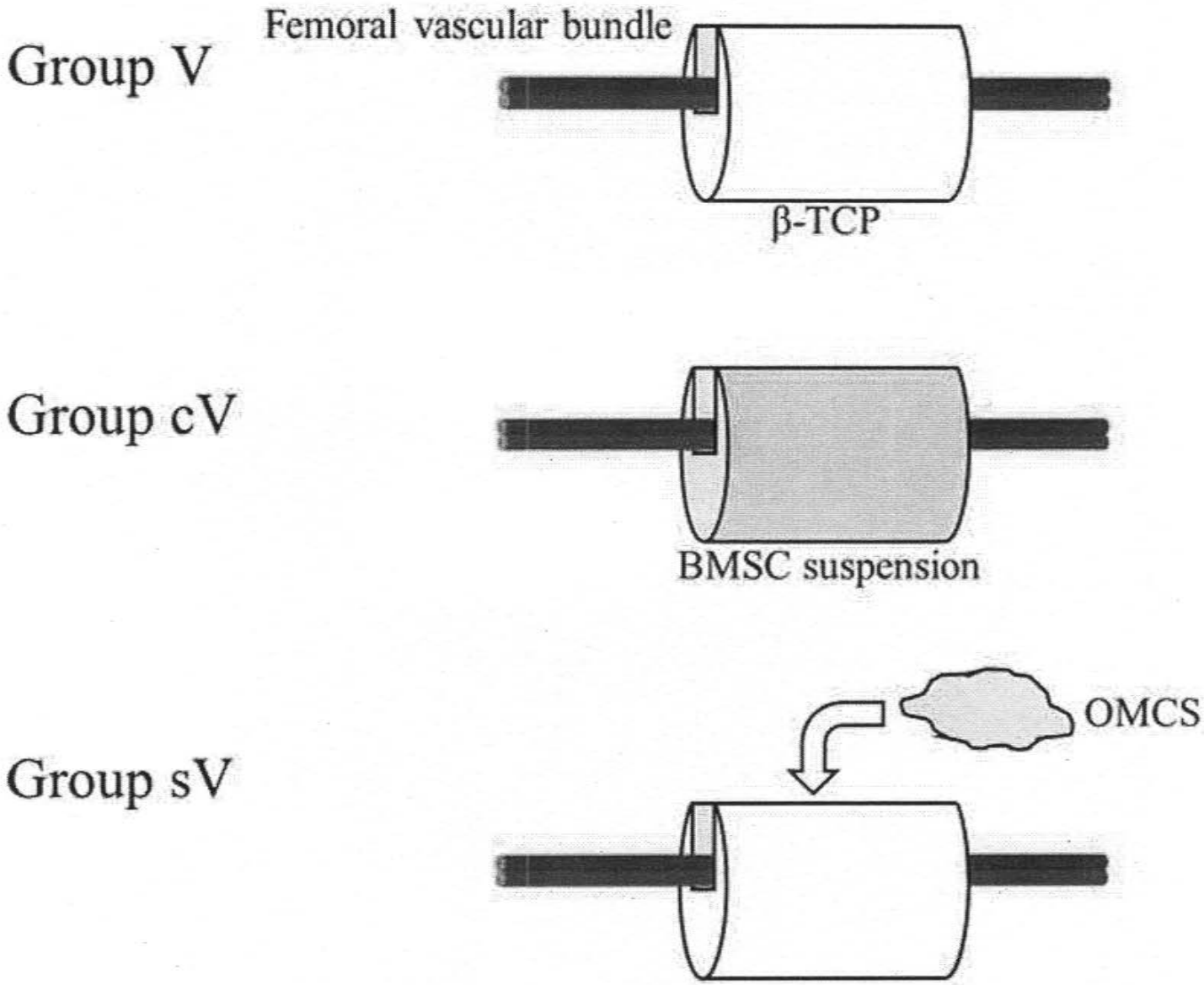

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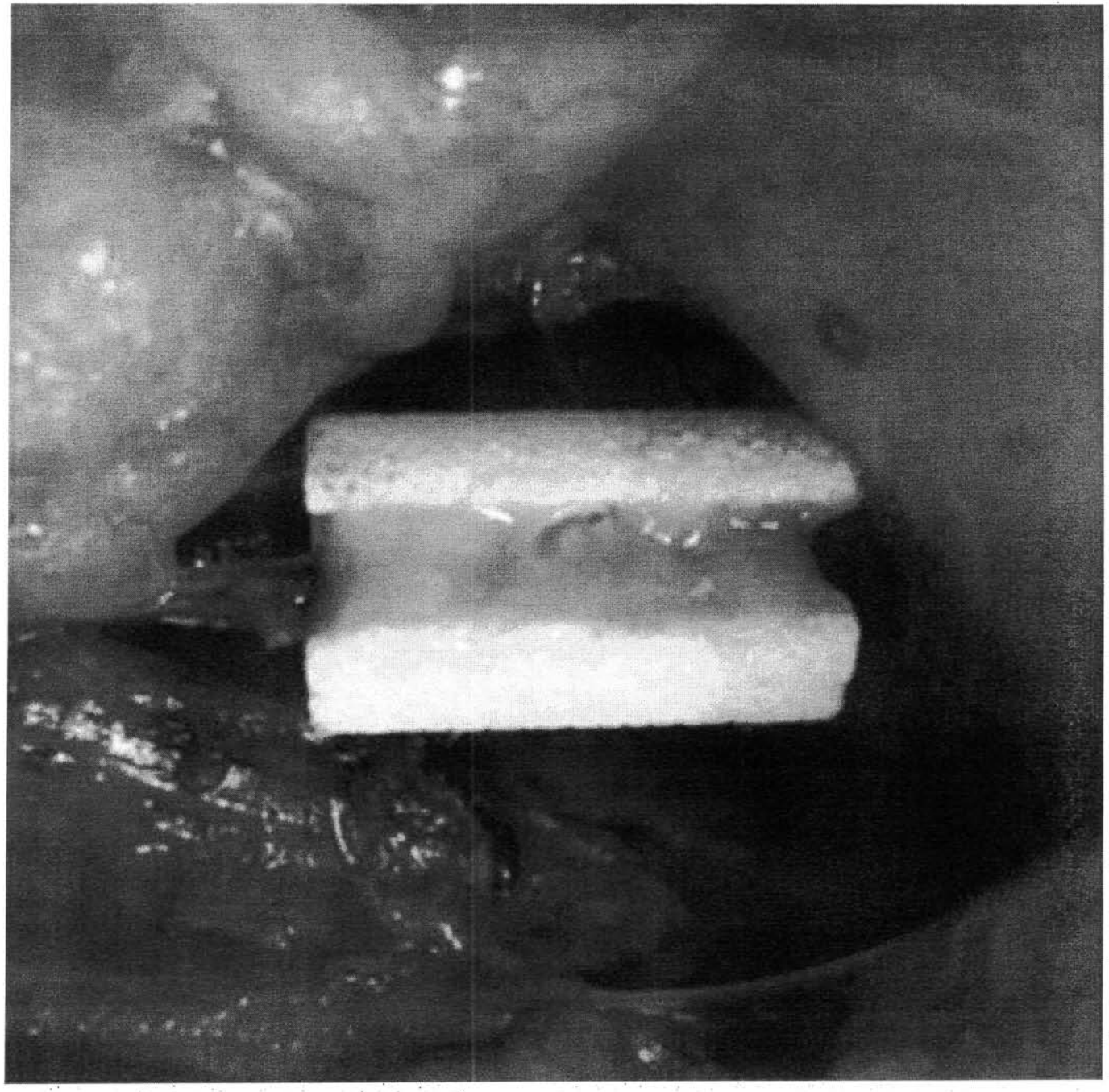

Figure3ab
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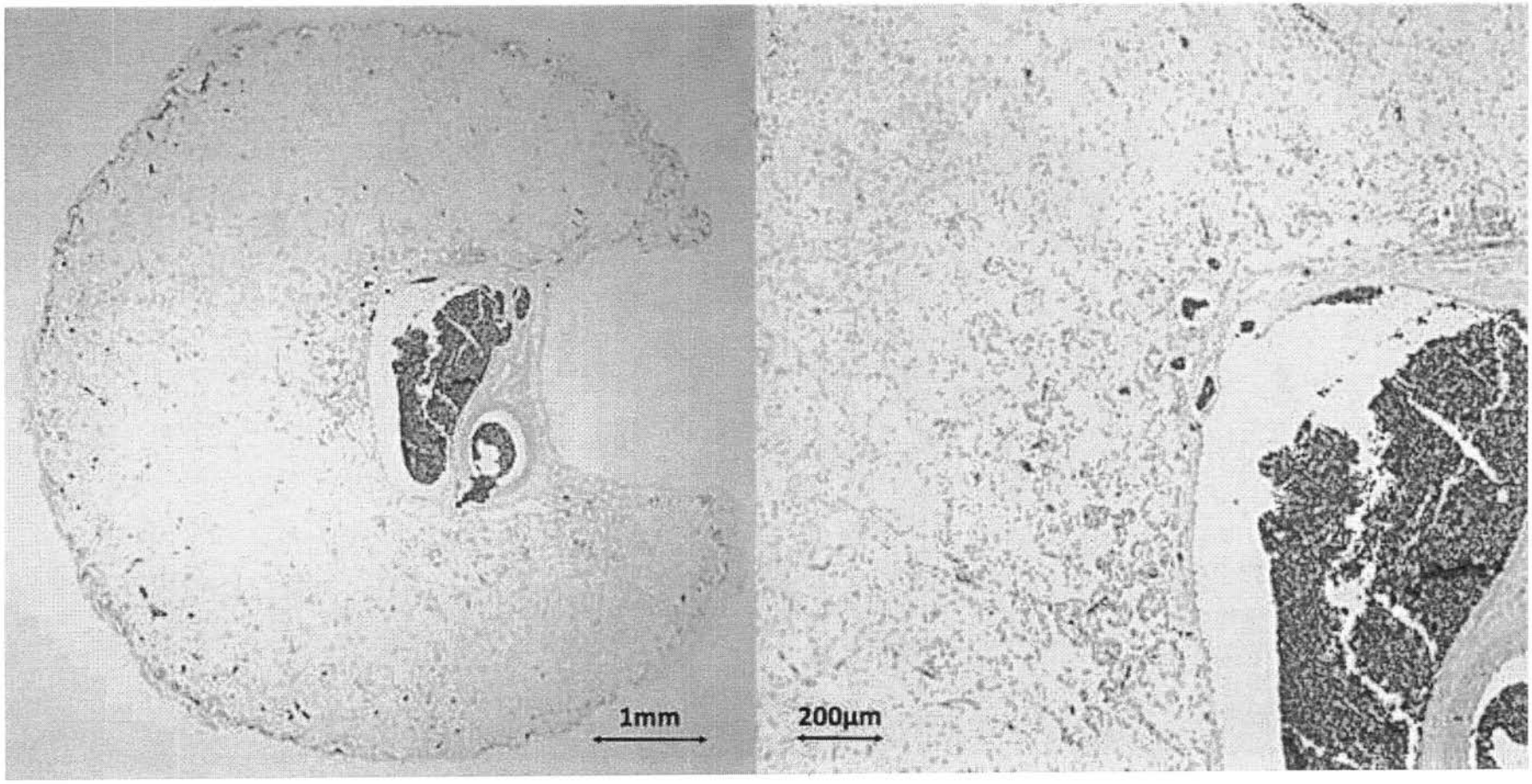

Figure3cd
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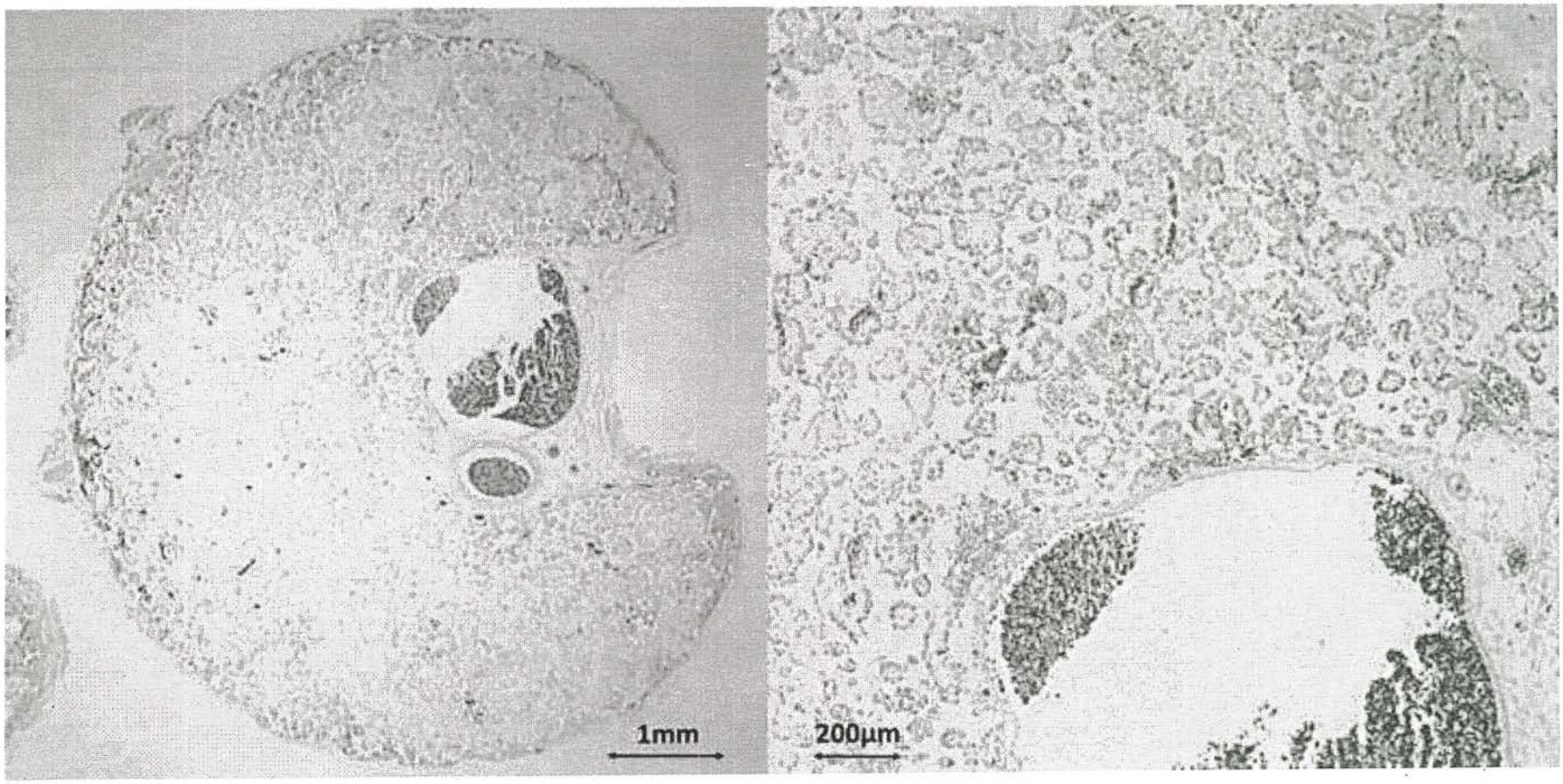

Figure3ef
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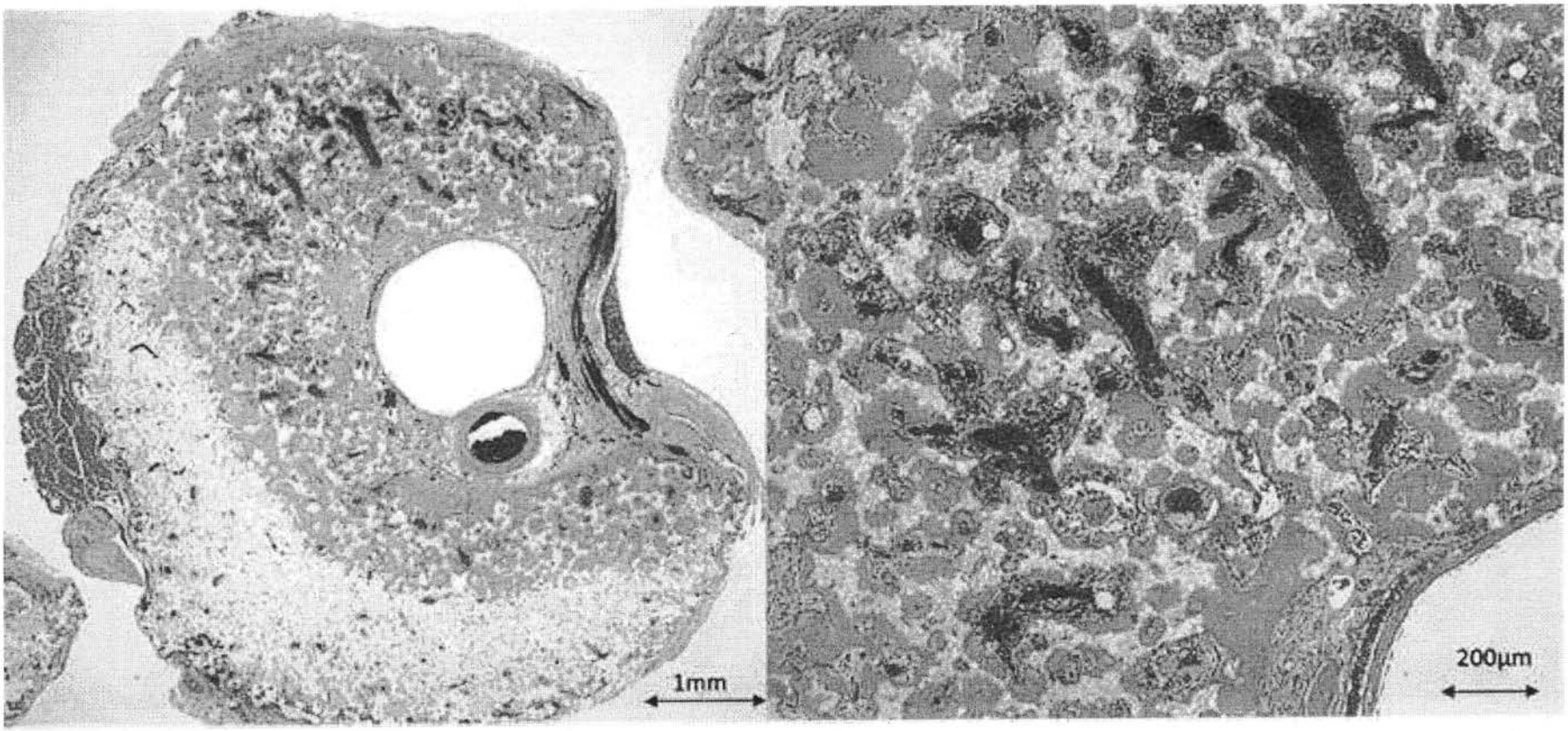

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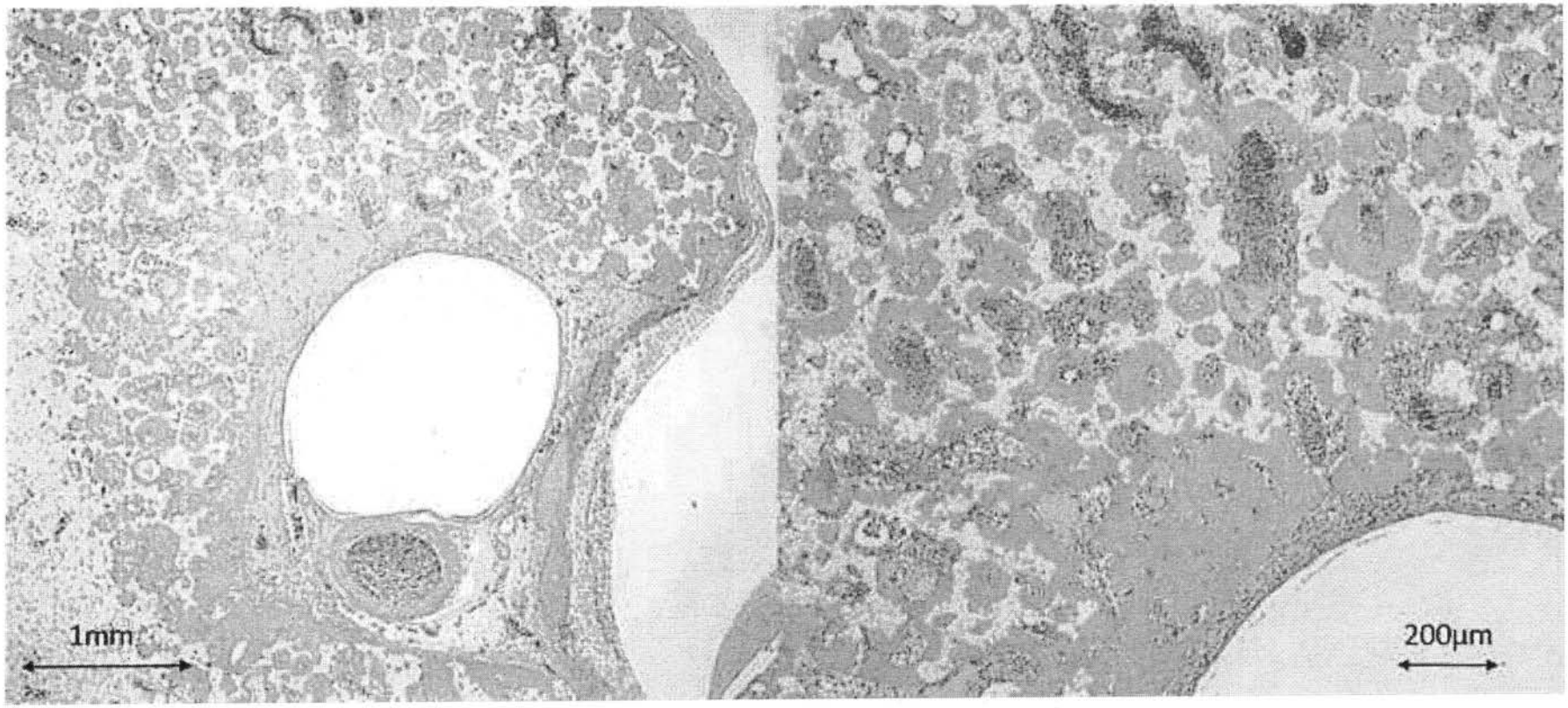

Figure4cd
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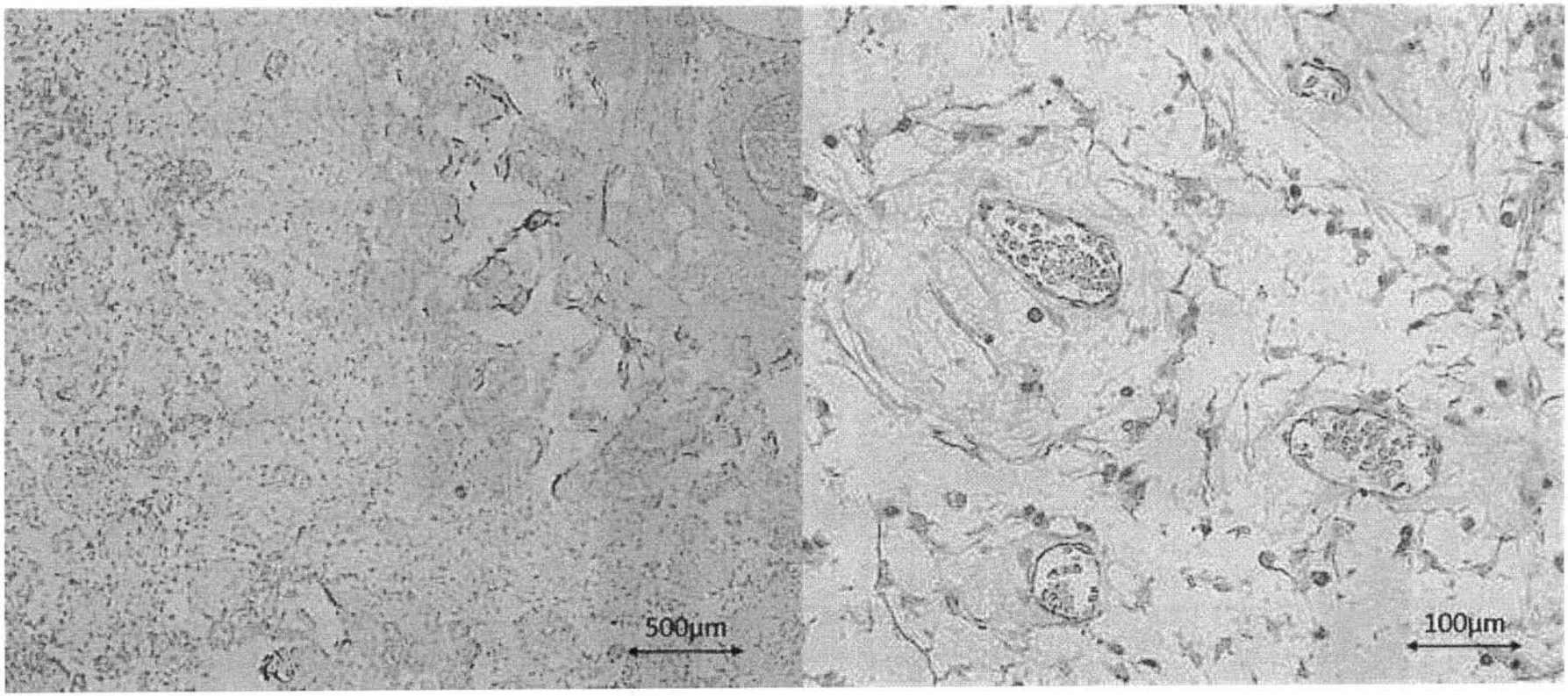

Figure5
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