

Influence of different additives and their concentrations on corneal toxicity and antimicrobial effect of benzalkonium chloride

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Key words : benzalkonium chloride, polyoxyethylene hydrogenated castor oil 40, polysorbate 80, cornea, antimicrobial, transepithelial electrical resistance

Abstract

Purpose: To examine the ophthalmic additives responsible for modulating acute corneal epithelial toxicity induced by benzalkonium chloride (BAC), and investigate the ability of polyoxyethylene hydrogenated castor oil 40 (HCO-40) and polysorbate 80 (PS-80) to reduce the corneal toxicity and antimicrobial effects of BAC.

Methods: Cytotoxicity of the additives, which included glycerin, polyvinyl alcohol, propylene glycol, polyethylene glycol and PS-80, on rabbit corneal epithelial cells was examined by the cell proliferation assay in the presence and absence of 0.02% BAC. Corneal transepithelial electrical resistance change after a 60 second exposure to HCO-40 or PS-80 mixed with 0.02% BAC was measured in living rabbits. Corneal damage was examined by scanning electron microscopy (SEM). The antimicrobial activities of HCO-40 and PS-80 with 0.02% BAC against *Staphylococcus aureus*, *Propionibacterium acnes*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Streptococcus pneumoniae* were assessed.

Results: Out of all the tested additives, only PS-80 could prevent the BAC-induced cytotoxicity. Corneal epithelial barrier function disorder caused by 0.02% BAC was significantly alleviated by either 0.1% and 1% PS-80 or HCO-40 in a concentration-dependent manner. SEM images showed improvement of BAC-induced corneal epithelial toxicity after addition of HCO-40 or PS-80. The antimicrobial effect of the BAC against *Pseudomonas aeruginosa*, *Escherichia coli* and

Streptococcus pneumoniae was reduced after adding HCO-40 or PS-80.

Conclusion: HCO-40 and PS-80 reduce acute corneal toxicity and the antimicrobial effect of BAC.

Possible interactions between BAC and other additives should be taken into consideration when evaluating the toxicity and antibacterial properties of BAC.

Key words: benzalkonium chloride, polyoxyethylene hydrogenated castor oil 40, polysorbate 80, cornea, antimicrobial, transepithelial electrical resistance

Introduction

Topical ophthalmic medications are the most prevalent and important therapy for ocular diseases. However, adverse ocular effects can occur from the preservatives used in these medications. The most commonly used preservative for eyedrops is benzalkonium chloride (BAC), which is a quaternary ammonium cationic surfactant. Although BAC is an effective preservative, previous studies have revealed it can cause ocular surface toxicities that include destabilization of the tear film¹, decreases in the corneal epithelial cell viability²⁻⁴, morphological changes in the corneal epithelial cells⁵⁻⁸, and disruption of the corneal epithelial barrier function^{9,10}. Furthermore, BAC has also been shown to induce precorneal tear film instability on the corneal surface and the appearance of dry spots¹¹. Clinical studies in healthy volunteers that compared the ocular tolerance of eyedrops with and without BAC demonstrated that there was induction of tear film instability or corneal barrier disruption in the subject group administered eyedrops with BAC compared with the BAC-free group^{12,13}. Therefore, chronic use of eyedrops containing BAC can potentially have an adverse effect on the cornea. In previous studies, after developing a new *in vivo* method for measuring the transepithelial electrical resistance (TER) of live rabbit corneas, we demonstrated that BAC concentrations between 0.005% - 0.02% immediately caused acute corneal barrier dysfunction^{14,15}. Furthermore, by using this method, it was demonstrated that polyoxyethylene hydrogenated castor oil 40 (HCO-40) as an additive suppressed BAC-induced corneal barrier dysfunction.¹⁶

Overall, these findings suggest that when evaluating drug toxicity, it is necessary to also consider potential interactions that could occur with the additives commonly found with BAC. Moreover, the previous findings have led us to suspect that the additives by themselves could be suppressing the antibacterial effect of BAC at the same time. Therefore, the aims of this study were first, to investigate whether or not common additives in eyedrops interact with BAC and then second, determine if these additives reduce the corneal toxicity. We specifically studied the effect of two candidate solutions (HCO-40 and PS-80) on the corneal toxicity and antimicrobial effects of BAC.

Materials and Methods

Chemicals

BAC 10% solution, glycerin, and PS-80 were obtained from Wako Pure Chemical Co., (Osaka, Japan). Polyvinyl alcohol (with an average degree of polymerization of 500), propylene glycol, and polyethylene glycol (with an average molecular weight of 400) were purchased from Nacalai Tesque, Inc., (Kyoto, Japan). HCO-40 was purchased from Nikko Chemicals, (Tokyo, Japan). 2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium monosodium salt (WST-1) was obtained from Dojindo Laboratories, Inc., (Kumamoto, Japan). Rabbit corneal epithelial cell growth medium (RCGM) and culture reagents were purchased from Kurabo Co. Ltd., (Osaka, Japan). Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution (HBSS) was obtained from Invitrogen Corp. (Carlsbad, CA,

USA). Test solutions such as 0.02% BAC, 1% glycerin, 1% polyvinyl alcohol, 1% propylene glycol, 1% polyethylene glycol, 0.01% - 1% HCO-40, and 0.01% - 1% PS-80 were prepared in RCGM or HBSS.

Cytotoxicity assay

Cytotoxicity of the additives on the NRCE cells was examined by a WST-1 assay in the presence and absence of 0.02% BAC. Normal rabbit corneal epithelial (NRCE) cells at the second passage were obtained from Kurabo Co., Ltd., (Osaka, Japan). The NRCE cells were maintained in RCGM, which was supplemented with 5 µg/mL insulin, 10 ng/mL epidermal growth factor, 0.5 µg/mL hydrocortisone, 50 µg/mL gentamicin, 50 ng/mL amphotericin B, and 0.4% bovine pituitary extract. The cells were grown at 37°C in a humidified atmosphere with 5% CO₂. Cytotoxicity tests on NRCE cells were carried out using a WST-1 commercially available cell proliferation reagent. NRCE cells were plated at 3×10^3 cells/well in 96-well microtiter plates (Becton-Dickinson, Franklin, NJ, USA). Six days after plating, the growth medium was replaced with 100 µL of the test solutions for 60 seconds. Cells were then washed with fresh growth medium, followed by addition of 100 µL of fresh growth medium and 10 µL of WST-1 mixture solution to each well. After incubation of the cells for 3 hours at 37°C, a microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to measure the absorbance at a wavelength of 450 nm, with a reference wavelength of 630 nm. The

results are presented as a percentage of the untreated cells.

Experimental animals

Male white Japanese rabbits (KBT Oriental, Tosu, Japan) weighing 2.5-3.0 kg were individually housed in cages under a controlled temperature (21°C) and humidity (50 ± 5%) and a 12:12 h light/dark cycle at the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine. Initiation of the study occurred once the rabbits reached weights of 3.0-4.0 kg, as this was the point where the corneal diameters were of a suitable size for experimentation. The rabbits were treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal TER measurement in vivo

The rabbits were anesthetized with an intramuscular injection of 30 mg/kg ketamine (Ketalar, Sankyo, Tokyo, Japan) and 5 mg/kg xylazine (Celactal, Bayer HealthCare, Osaka, Japan). After a small incision was made with an 18-gauge sharp needle (Terumo, Tokyo, Japan) in the peripheral cornea, a 1.0-mm diameter custom-made Ag/AgCl electrode (Physiotech, Tokyo, Japan) was inserted into the anterior chamber. A 6.0 mm internal diameter (0.28 cm² inner area) nitrile rubber O-ring (Union Packing, SAN-EI, Osaka, Japan) was fixed on the cornea using biomedical adhesive

(Alon-Alpha A, Sankyo, Tokyo, Japan). Subsequently, 80 μ L of HBSS was placed inside the ring, with the second electrode then placed in HBSS on the cornea. The TER was measured in real time using a volt-ohm meter (EVOMX, World Precision Instruments, Sarasota, FL, USA). In a period of just a few seconds, 1 mL of the test solutions was gently poured into the ring, with all of the overflow aspirated. After an exposure period of 60 seconds, the rings were washed out using 1 mL of HBSS. After obtaining the TER of the cornea before and after the exposure, results were then calculated as a percentage of the pre-exposure TER value (100%) (n=3-4). This specific methodology and photographs of the *in vivo* corneal TER measurement system have been previously published¹⁴⁻¹⁶. In this study, the influence of the two candidate additives (HCO-40 and PS-80) on the BAC-induced corneal TER changes was analyzed by examining different concentrations of each additive in the presence of 0.02% BAC. The sample size for the corneal TER study was set at 3 to 4, which we found to be sufficient for our statistical analyses in our previous TER studies¹⁴⁻¹⁶.

Scanning electron microscopy (SEM) observation

The rabbits were anesthetized with an intramuscular injection of 30 mg/kg ketamine and 5 mg/kg xylazine. Corneas were evenly soaked in the test solution for 60 seconds. After the corneal washing, the rabbits were immediately sacrificed using a lethal dose of intravenous sodium pentobarbital (Nembutal, Dainippon Pharmaceutical, Osaka, Japan). The corneas were carefully excised, fixed in

4% glutaraldehyde in 0.05 M cacodylate buffer for 1 hour and then post-fixed in 1% osmium tetroxide in veronal acetate buffer containing 0.22 M sucrose. The fixed materials were dehydrated through a series of ethanol washes. Corneas were placed in *t*-butyl alcohol, treated in a freeze-drying apparatus (EIKO ID-2, EIKO, Tokyo, Japan), and then sputter-coated with gold using an auto fine coater (JEOL JFC-1600, JEOL, Tokyo, Japan). After processing, the surface of the corneal epithelium was observed by a scanning electron microscope (Hitachi S2360, Hitachi, Ibaragi, Japan).

Minimum inhibitory concentration determination

Minimum inhibitory concentrations (MICs) of the agents against bacteria that cause ocular surface infections were determined by the standard macro-dilution assay. *Staphylococcus aureus* (ATCC 29213), *Propionibacterium acnes* (ATCC6919), *Pseudomonas aeruginosa* (ATCC27853), and *Escherichia coli* (ATCC25922) were examined according to the Method of Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Eighth Edition (CLSI document M07-A8, 2008; CLSI, Wayne, PA, USA). *Streptococcus pneumoniae* (ATCC49619) was examined according to the Performance Standards for Antimicrobial Susceptibility Testing; Twentieth Informational Supplement (CLSI document M100-S20, 2010; CLSI). *Propionibacterium acnes* was examined according to the Methods for Antimicrobial Susceptibility Tests of Anaerobic Bacteria; Approved Standard-Seventh Edition (CLSI document

M11-A7, 2007, CLSI). In brief, bacterial cultures were adjusted to an optical density of the 0.5 McFarland standard. After being diluted in the solutions, the final inoculum was approximately 10^5 CFU/well. The solutions used were 0.02% BAC alone or 0.02% BAC mixed with either HCO-40 or PS-80, with the final concentrations set at 0.01%, 0.03%, 0.1%, 0.3%, and 1%. The lowest concentration of agent that prevented visible growth was considered as the MIC.

Statistical analysis

All results were expressed as the mean \pm standard error of at least three experiments. Statistical comparisons were performed using an analysis of variance followed by a Tukey-Kramer test for the TER measurement and cytotoxicity assay. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

Cytotoxicity assay

The cytotoxicity assay for each additive was performed individually. BAC exhibited low cell viability (5 ± 2 %). However, glycerin (71 ± 20 %), polyvinyl alcohol (121 ± 34 %), propylene glycol (110 ± 19 %), polyethylene glycol (102 ± 19 %), and PS-80 (127 ± 28 %) showed cell

viability that was comparable to the control cells (Fig. 1A). In the presence of BAC, PS-80 did not show cytotoxicity ($92 \pm 17 \%$), although glycerin ($5 \pm 2 \%$), polyvinyl alcohol ($5 \pm 1 \%$), propylene glycol ($6 \pm 1 \%$), and polyethylene glycol ($5 \pm 1 \%$) exhibited significantly lower cell viability as compared with PS-80 (Fig. 1B).

TER change

After 0.02% BAC exposure the TER significantly decreased to $10 \pm 8\%$ of the initial value compared to the control ($97 \pm 3\%$). In the presence of 0.02% BAC, adding 0.01% HCO-40 or PS-80 did not significantly improve the corneal barrier function. At this concentration, the corneal TER was $11 \pm 5\%$ and $21 \pm 4\%$ of the baseline, respectively. However, 0.1% HCO-40 or PS-80 mixed with 0.02% BAC increased the corneal TER to $50 \pm 10\%$ and $58 \pm 11\%$ of the initial value, respectively. After adding 1% HCO-40 or PS-80 with 0.02% BAC, there was significant improvement of the corneal TER to $96 \pm 4\%$ and $96 \pm 7\%$ of the initial value, respectively (Fig. 2A, 2B).

Scanning electron microscopy observation

After exposure to control HBSS, the SEM showed that the superficial cells were intact with normal microvilli (Fig. 3A). Conversely, after exposure to 0.02% BAC, the superficial cells were damaged and exhibited degenerated microvilli (Fig. 3B). After adding the 1% HCO-40 or PS-80

mixed with 0.02% BAC, there were no changes in the appearance of the superficial cells (Fig. 3C, 3D). After adding 0.1% HCO-40 or PS-80 mixed with 0.02% BAC, superficial cells were mildly injured. (Fig. 3E, 3F). After adding 0.01% HCO-40 or PS-80 mixed with 0.02% BAC, damaged superficial cells with degenerated microvilli were observed (Fig. 3G, 3H).

MIC determination

The MIC experiments demonstrated that 0.01% HCO-40 or 0.03% PS-80 maintained the preservative effect of 0.02% BAC. In contrast, growth of *Pseudomonas aeruginosa* in the culture medium was observed with concentrations of more than 0.03% HCO-40 or 0.1% PS-80 in the presence of 0.02% BAC. Growth of *Escherichia coli* and *Streptococcus pneumoniae* in the culture medium was also observed with concentrations of 1% HCO-40 or PS-80 in the presence of 0.02% BAC (Table 1A, 1B).

Discussion

Several compounds such as hyaluronic acid have been shown to exert a protective effect against BAC-induced ocular toxicity¹⁷⁻²¹. Furthermore, we have previously demonstrated that HCO-40 interacts with BAC and reduces corneal toxicity¹⁶. Possible interactions between BAC and other ophthalmic additives should be taken into consideration when evaluating BAC-induced toxicity in

eyedrops. In the current study, we investigated whether or not general ophthalmic additives could influence corneal toxicity caused by BAC. Furthermore, since it is also suspected that these BAC-inhibiting additives may simultaneously suppress the antibacterial effect of BAC, we studied the effect of two candidate solutions (HCO-40 and PS-80) on both the corneal toxicity and antimicrobial effects of BAC.

Additives were prepared in the presence or absence of 0.02% BAC, which is the highest concentration currently utilized in commercial eyedrops. As normally tears dilute the eyedrops immediately after instillation,²²⁻²⁴ our study was designed to examine the corneal exposure over a 60-second period. In a previous study, our results revealed that the cytotoxicity assay is useful for estimating corneal epithelial change¹⁵. Thus, in the current study we used NRCE cells to initially confirm that the corneal effect was induced by ophthalmic additives with or without BAC. After exposure to BAC, the NRCE cells exhibited a significantly lower viability, although exposure to glycerin, polyvinyl alcohol, propylene glycol, polyethylene glycol, and PS-80 did not affect the cell viability. In the presence of BAC, cytotoxicity was still observed with the addition of glycerin, polyvinyl alcohol, propylene glycol, and polyethylene glycol, which suggests that these additives did not suppress the BAC-induced corneal impairment. However, after exposure to PS-80 with BAC, no significant changes were noted in the cell viability. Similar to these results, our previous study also showed that HCO-40 interacted with BAC to reduce the corneal toxicity¹⁶. Therefore, we performed

further experiments using an *in vivo* electrophysiological method to examine the influence of HCO-40 and PS-80 on BAC in living rabbit corneas.

We previously developed a new *in vivo* corneal TER measurement system that makes it possible to continuously and quantitatively measure corneal changes within only a few seconds¹⁴. In general, TER reflects the barrier function of the epithelium, with lower corneal TER values indicative of the penetration of greater amounts of electrical current through the damaged superficial cells and tight junctions existing in the epithelium^{9,10,14-16}. In the current study the results of the TER measurement showed that both HCO-40 and PS-80 remarkably prevented the BAC-induced acute corneal barrier dysfunction in a concentration-dependent manner. This protective effect of HCO-40 and PS-80 against BAC was further confirmed by histological analysis using SEM. After exposure of the corneal epithelium to 0.02% BAC with 1% HCO-40 and 1% PS-80, our SEM-based histological analyses showed that the superficial layer of the cornea had a better appearance than the 0.02% BAC-treated corneal image. These results are in agreement with the above-mentioned findings. Overall, the present results demonstrate that HCO-40 and PS-80 are able to modulate BAC toxicity.

On the other hand, it has been suspected that these other additives may simultaneously suppress the antibacterial effect of BAC. Therefore, we also examined the effect of HCO-40 and PS-80 on the antimicrobial property of BAC against bacteria that can induce ocular surface infections. MIC determinations showed that HCO-40 and PS-80 reduced the MICs of the 0.02% BAC in

Pseudomonas aeruginosa, *Escherichia coli*, and *Streptococcus pneumoniae*. It should be particularly noted that in *Pseudomonas aeruginosa*, which can cause severe corneal infections, even lower concentrations of HCO-40 or PS-80 suppressed the antimicrobial effect of BAC. Based on these current findings, the question that remains to be answered is what mechanism enables HCO-40 and PS-80 to reduce BAC activity in the corneal epithelium and bacteria. HCO-40 and PS-80, which is also known as “TWEEN 80”, are both composed of hydrophobic portions and hydrophilic long hydrocarbon chains. In our previous study, we demonstrated that 1% HCO-40 formed micelles that had a 25.7 nm mean particle size with a slightly negative surface charge. When 0.02% BAC was added to the 1% HCO-40 micelles, this induced a significant reduction in particle size along with a modification of the surface charge from negative to positive¹⁶. These results indicate that the micelles of 1% HCO-40 with 0.02% BAC have a coating layer of BAC molecules. Both HCO-40 and PS-80 are used as a solubilizer in eyedrops and are classified as nonionic surfactants. Furthermore, a previous study using an *in vitro* TER system reported that the PS-80 contained in isopropyl unoprostone ophthalmic solution influenced the TER measurements due to interactions with the BAC found in the solution²⁵. This finding suggests that the modulation mechanism of HCO-40 and PS-80 may involve an interaction with BAC through hydrophobic bonding and ionic bonding, which can then lead to a decrease in the number of free BAC molecules that attach to the corneal epithelium or bacteria. This theory agrees with the previous study that showed use of

emulsion led to a reduction of the BAC-induced ocular surface toxicity²⁶. It has also been postulated that the suppressive mechanism could possibly be related to binding between BAC and an emulsion surface, which would then lead to a reduction in the amount of free BAC molecules²⁷. In our current study, we were able to confirm that polysorbate 20, which is analogous to PS-80, also improved the corneal barrier dysfunction (data not shown). This finding indicates that many compounds analogous to HCO and PS could have similar effects on both the toxicity and antimicrobial activity of BAC. Since HCO-40 and PS-80 are nonionic surfactants, they are also used as solubilizers in various products including ophthalmic solutions. Nonionic surfactants have been shown to be safer with regard to ocular irritation as compared with other classes of surfactants^{28,29}. In clinical studies of patients with dry eye symptoms, an emulsion containing PS-80 and castor oil, which is analogous to HCO-40, was shown to increase the tear film stability and reduce subjective symptoms, such as grittiness and burning³⁰. Therefore, HCO-40 and PS-80 may be effective additives that can be used in artificial tears. Moreover, these facts imply that there may be another mechanism by which HCO-40 and PS-80 can reduce the BAC toxicity on the corneal surface. Another possibility is that HCO-40 and PS-80 may not interact with BAC, but instead, they may exert a protective effect on the corneal epithelium itself that helps the corneal surface to resist the BAC toxicity. However, this effect has yet to be demonstrated in any currently published reports. Thus, further studies will be necessary to definitively elucidate the details of these possible mechanisms. The results of the

current study suggest that HCO-40 and PS-80 have a suppressive effect on BAC activity. While the use of these compounds may be good for the corneal surface, there is a chance that they may have a negative influence on the antimicrobial effects of BAC. At the present time, HCO-40 and PS-80 are often used with BAC in many types of liquid products. Therefore, when producing solutions such as eyedrops, possible interactions of BAC with additives must be considered in terms of both toxicity and antibacterial properties.

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Figure Legends

Fig. 1

Cytotoxicity assay of various ingredients in NRCE cells without (A) or with (B) 0.02% BAC. NRCE cells were exposed to solutions with varying ophthalmic ingredients for 60 seconds. Data represent the percentage compared to the untreated cells (control). BAC is the positive control. Each value is the mean \pm S.E. (n = 6). * $p < 0.05$, ** $p < 0.01$ as compared with BAC. ## $p < 0.01$ as compared with BAC, glycerin, polyvinyl alcohol, propylene glycol, and polyethylene glycol.

Fig. 2

Corneal TER changes after an exposure to 0.02% BAC with 0.01%, 0.1%, or 1% of HCO-40 (A) or PS-80 (B) for 60 seconds. Data represent the percentage compared to the pre-exposure value. HBSS served as the negative control while BAC served as the positive control. Each value is the mean \pm S.E. (n = 3-4). ** $p < 0.01$ as compared with 0.01% or 0.1% of either HCO-40 or PS-80. ## $p < 0.01$ as compared with HBSS.

Fig. 3

Scanning electron microscopy images of the corneal epithelium after 60 seconds exposure to HBSS (A), 0.02% BAC (B), 0.02% BAC with 1% HCO-40 (C), 0.02% BAC with 1% PS-80

(D), 0.02% BAC with 0.1% HCO-40 (E), 0.02% BAC with 0.1% PS-80 (F), 0.02% BAC with 0.01% HCO-40 (G), 0.02% BAC with 0.01% PS-80 (H) at 12000× magnification. Images A, C, and D show that the corneal epithelial structures remain almost intact. Images B, G, and H show injured corneal epithelial structures including degenerated microvilli. Images E and F show mildly injured corneal epithelial structures.

Table legend

Table. 1

Minimum inhibitory concentration determinations for 0.02% BAC mixed with HCO-40 (A) and PS-80 (B) on *Staphylococcus aureus*, *Propionibacterium acnes*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Streptococcus pneumoniae*. “-” indicates bacterial growth was negative. “+” indicates bacterial growth was positive.

TABLE 1. MIC determinations for 0.02% BAC mixed with HCO-40 (A) and PS-80 (B)

(A)	0.02% BAC					
	HCO-40					
	0.01%	0.03%	0.1%	0.3%	1%	
<i>S. aureus</i>	-	-	-	-	-	-
<i>P. acnes</i>	-	-	-	-	-	-
<i>P. aureginosa</i>	-	-	+	+	+	+
<i>E. coli</i>	-	-	-	-	-	+
<i>S. pneumoniae</i>	-	-	-	-	-	+

(B)	0.02% BAC					
	PS-80					
	0.01%	0.03%	0.1%	0.3%	1%	
<i>S. aureus</i>	-	-	-	-	-	-
<i>P. acnes</i>	-	-	-	-	-	-
<i>P. aureginosa</i>	-	-	-	+	+	+
<i>E. coli</i>	-	-	-	-	-	+
<i>S. pneumoniae</i>	-	-	-	-	-	+

Figure 1A

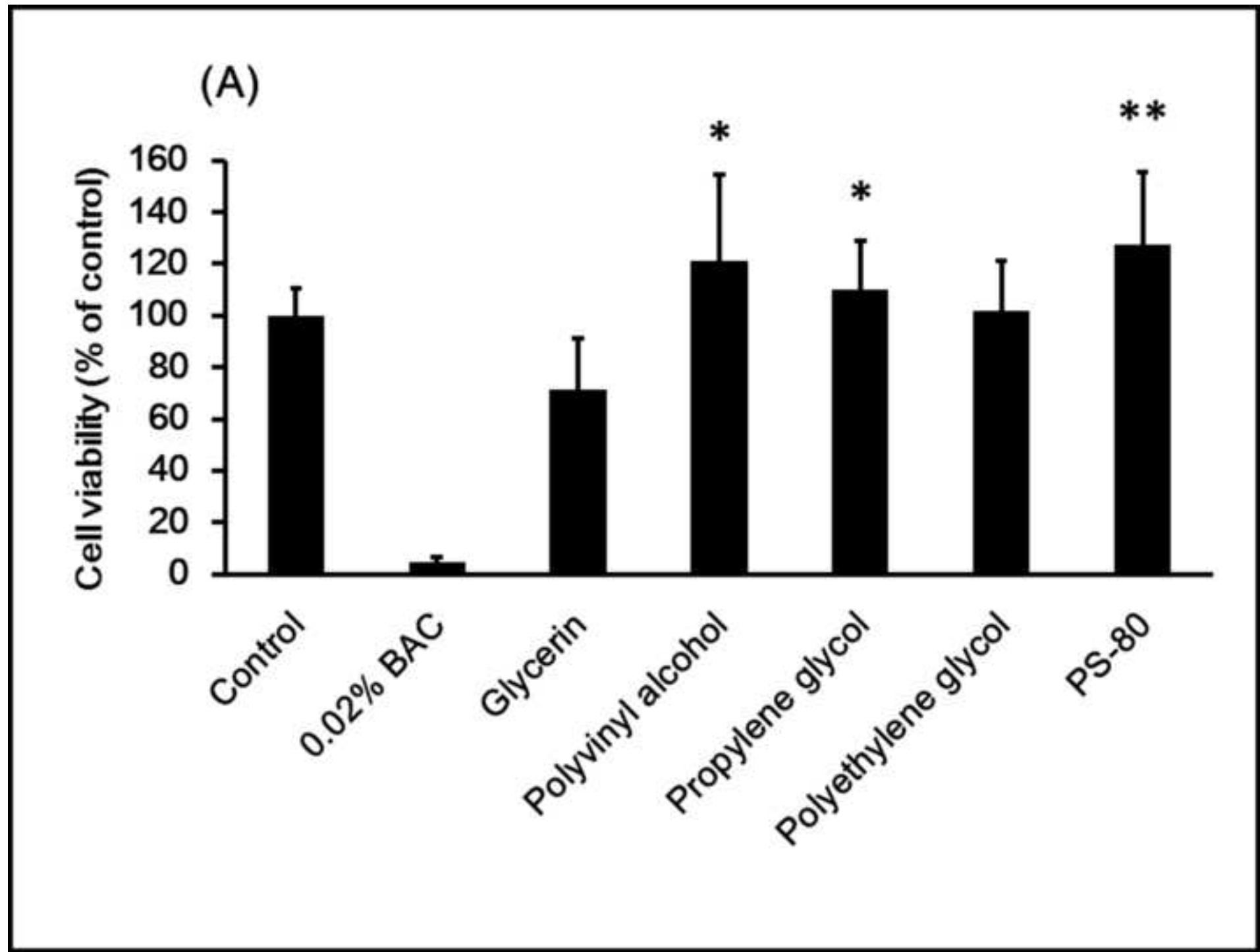


Figure 1B

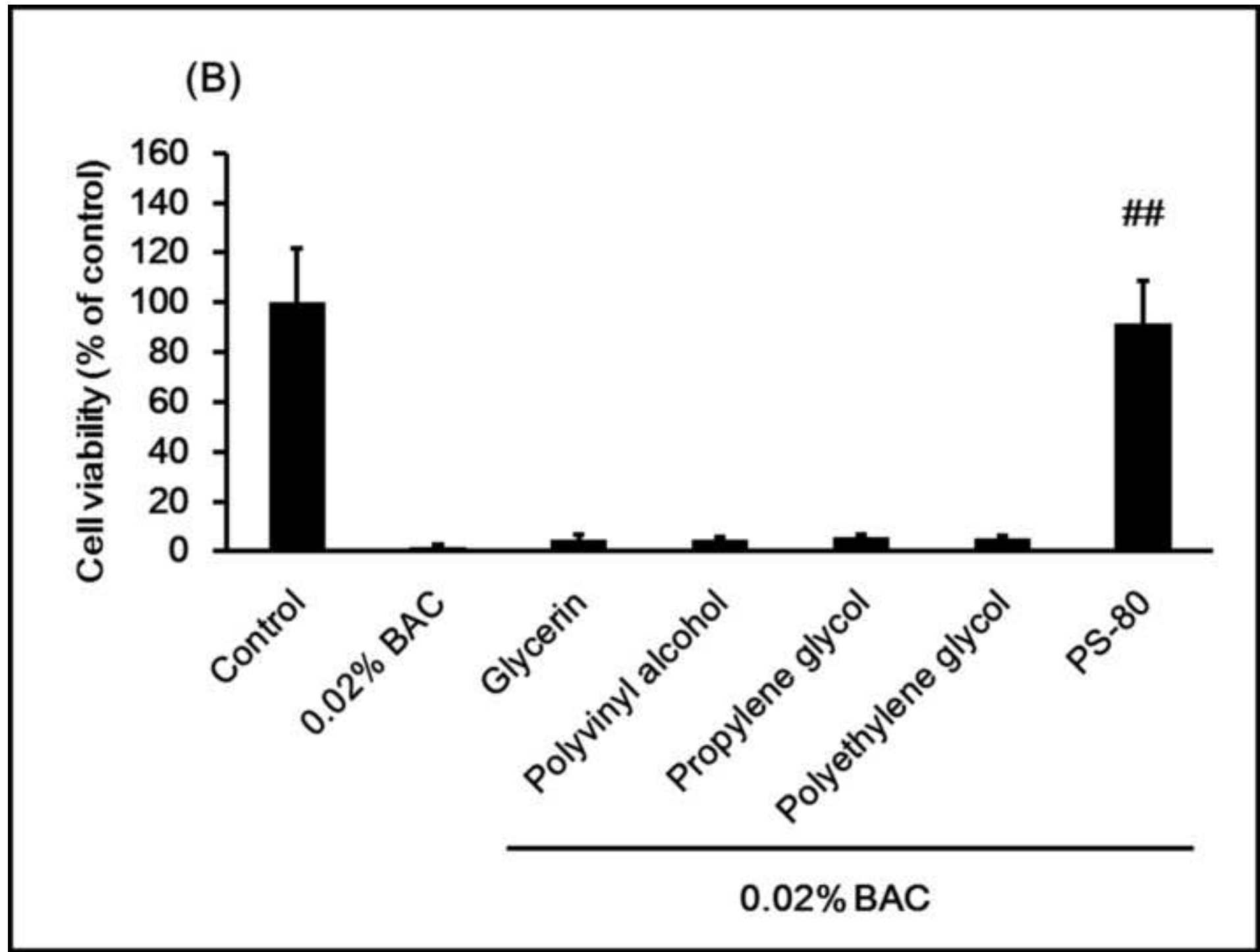


Figure 2A

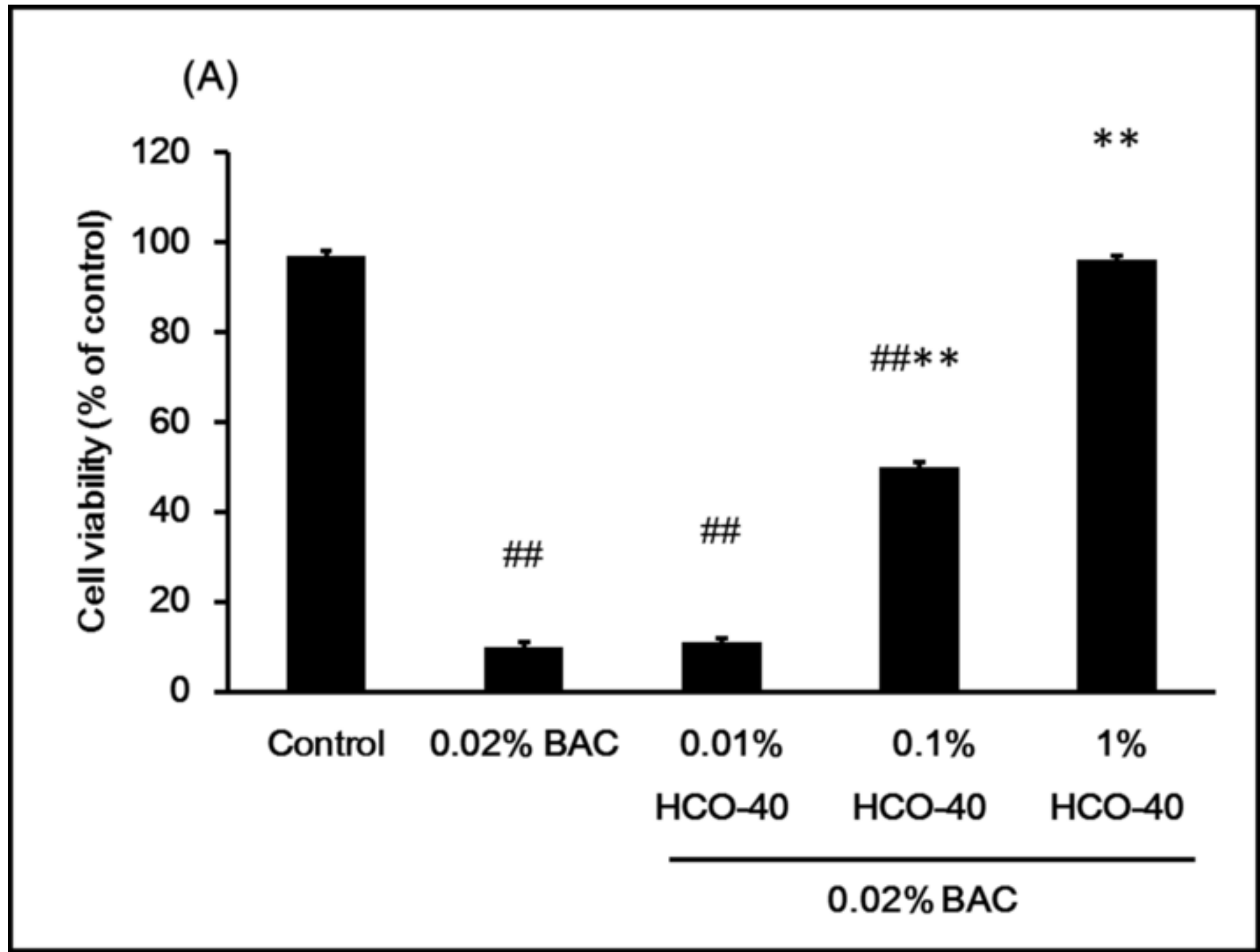


Figure 2B

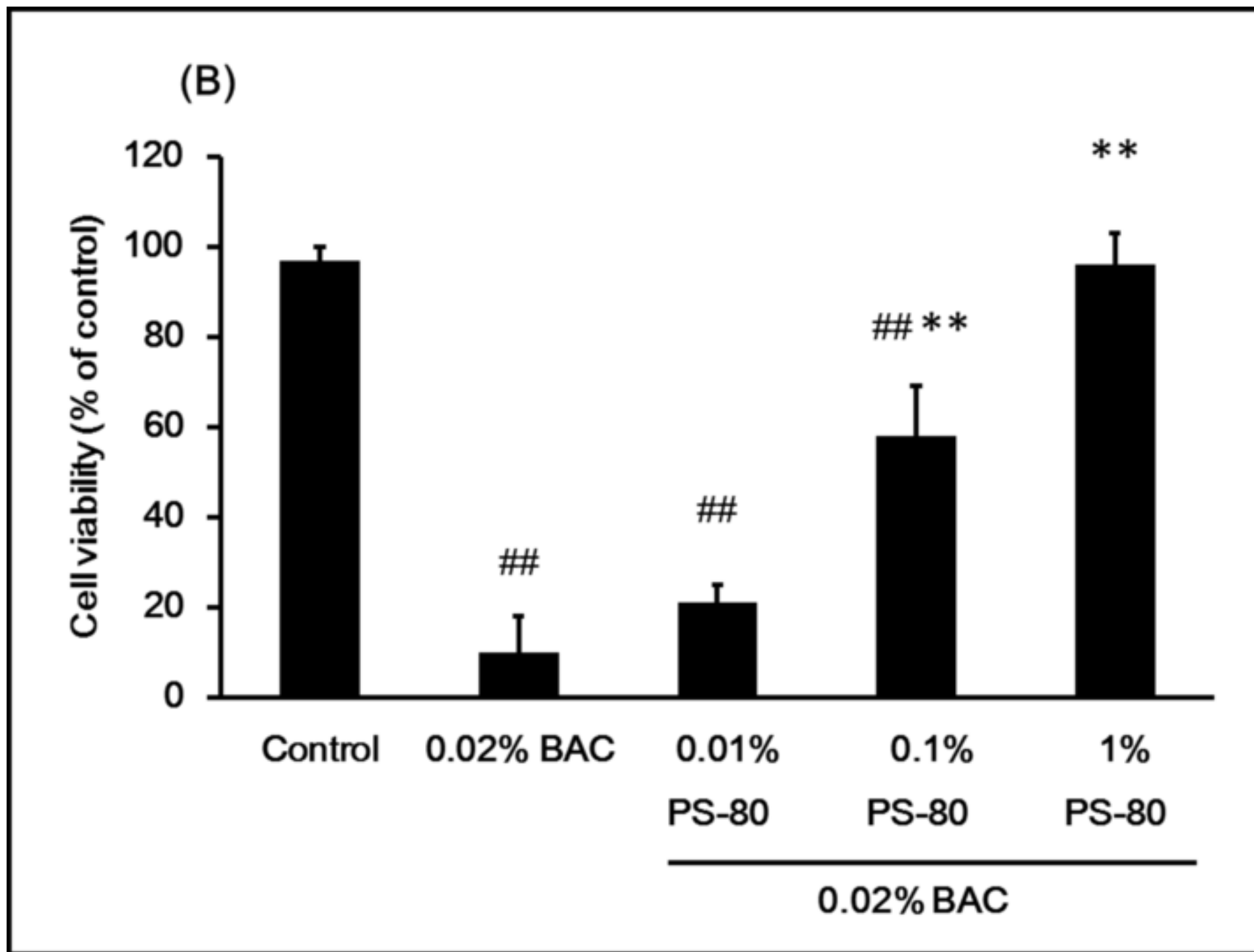


Figure 3A

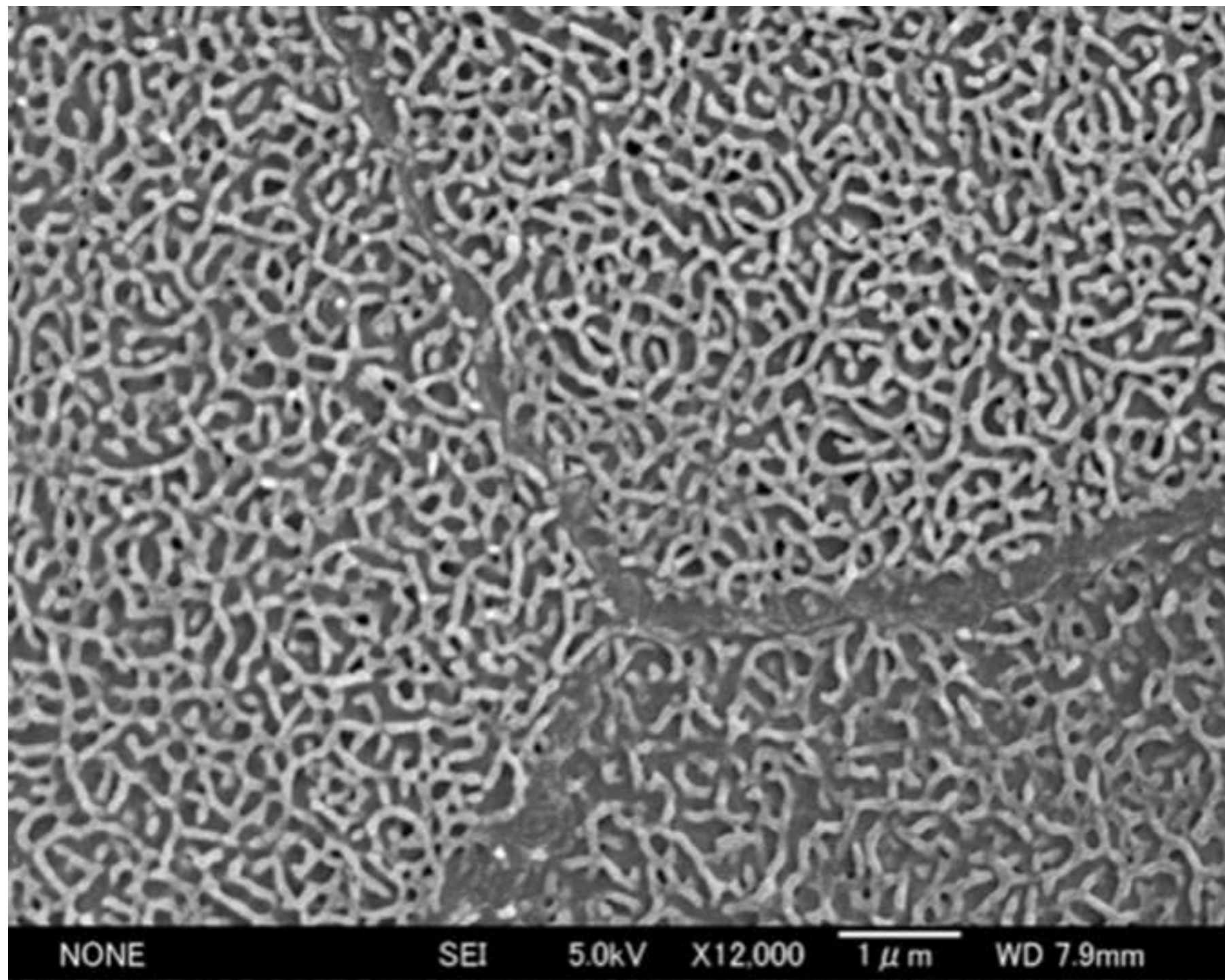


Figure 3B

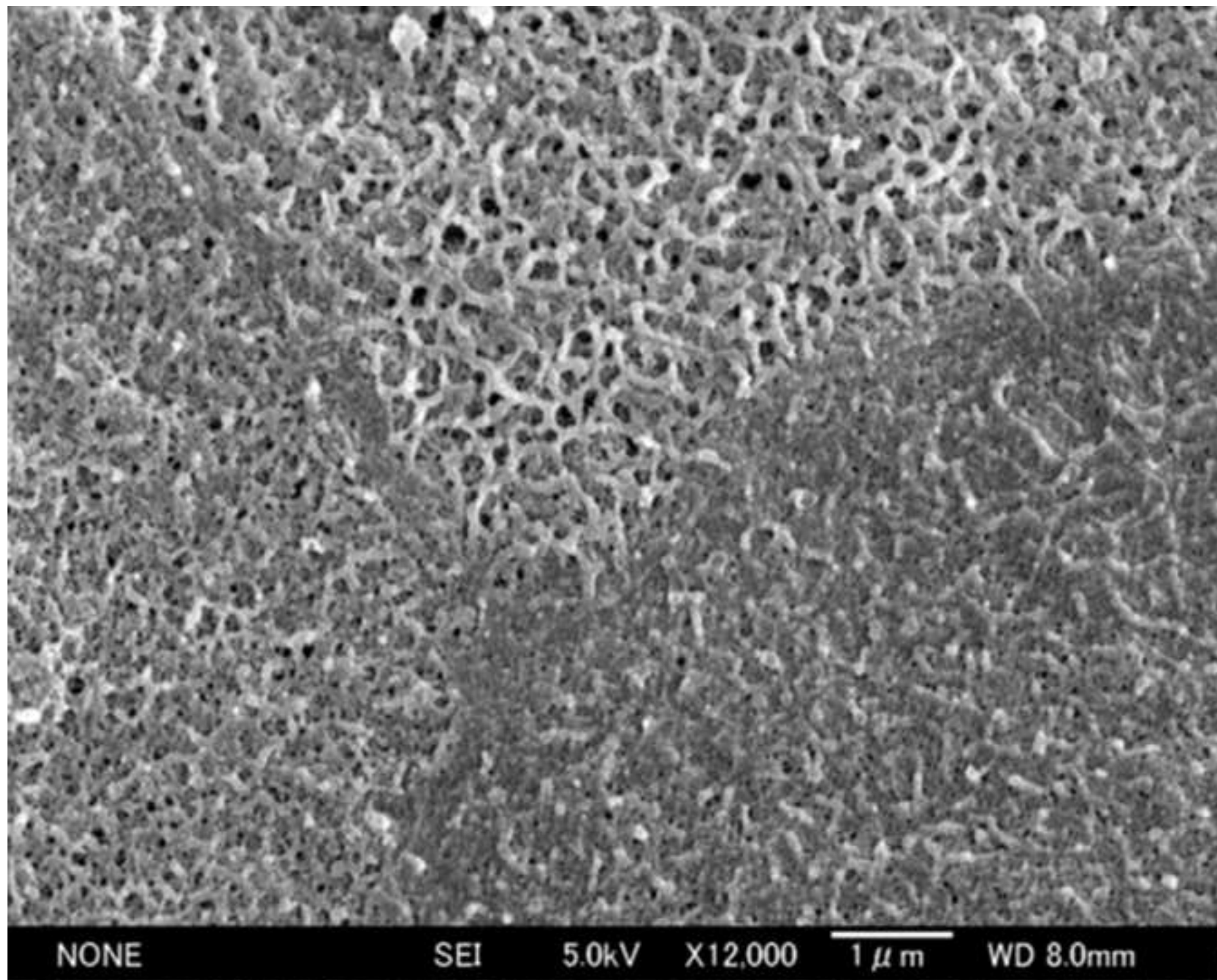


Figure 3C

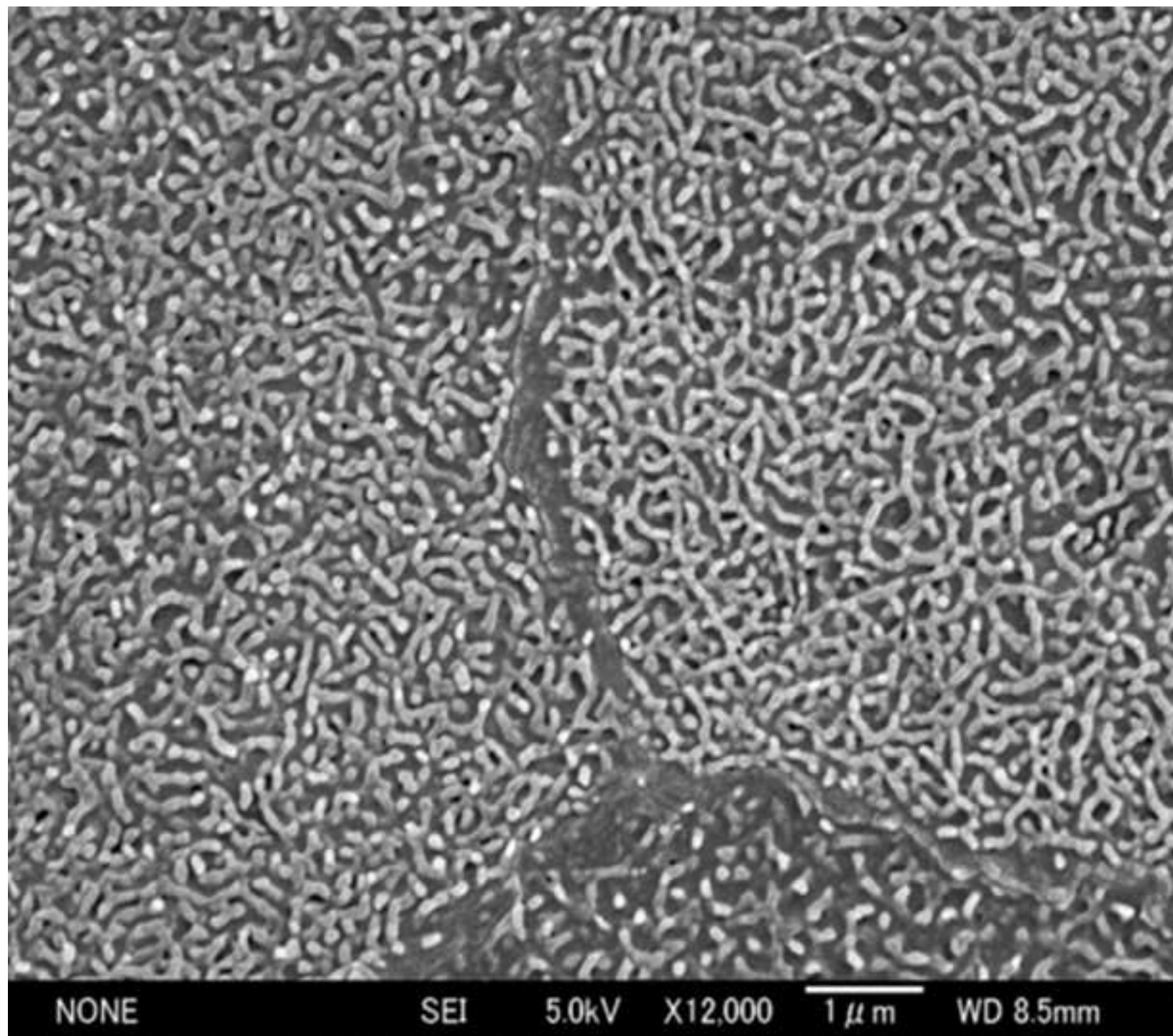


Figure 3D

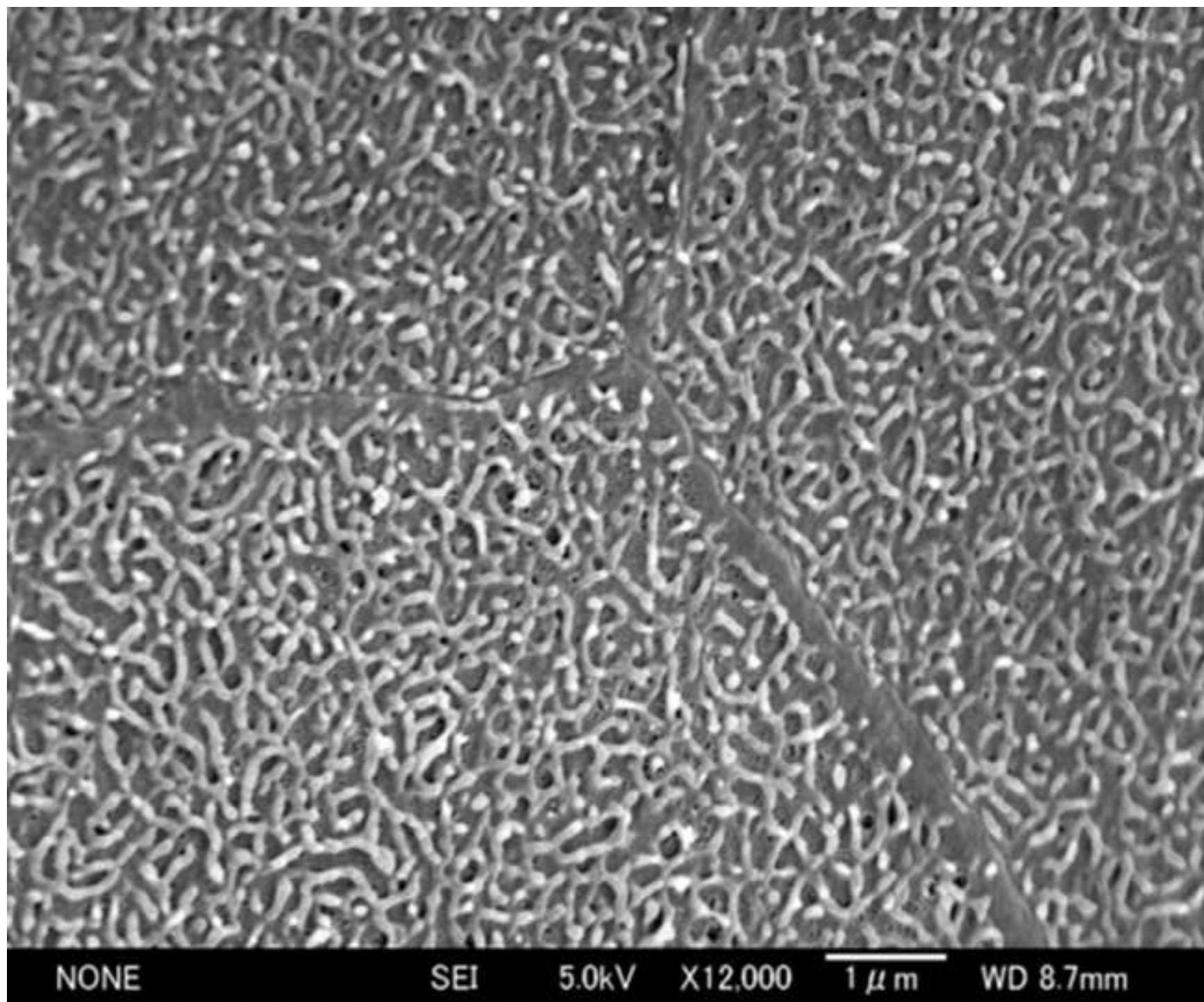


Figure 3E

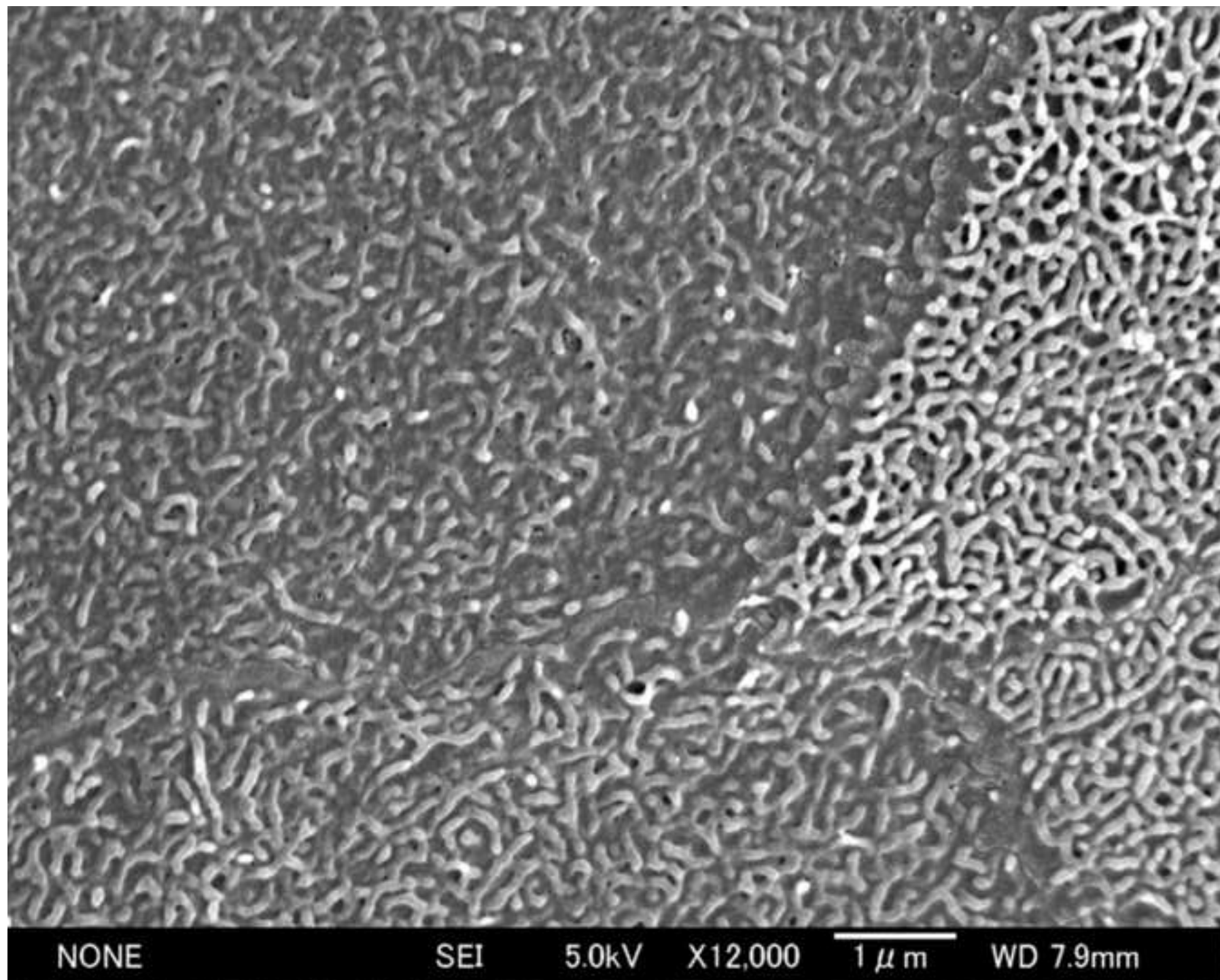


Figure 3F

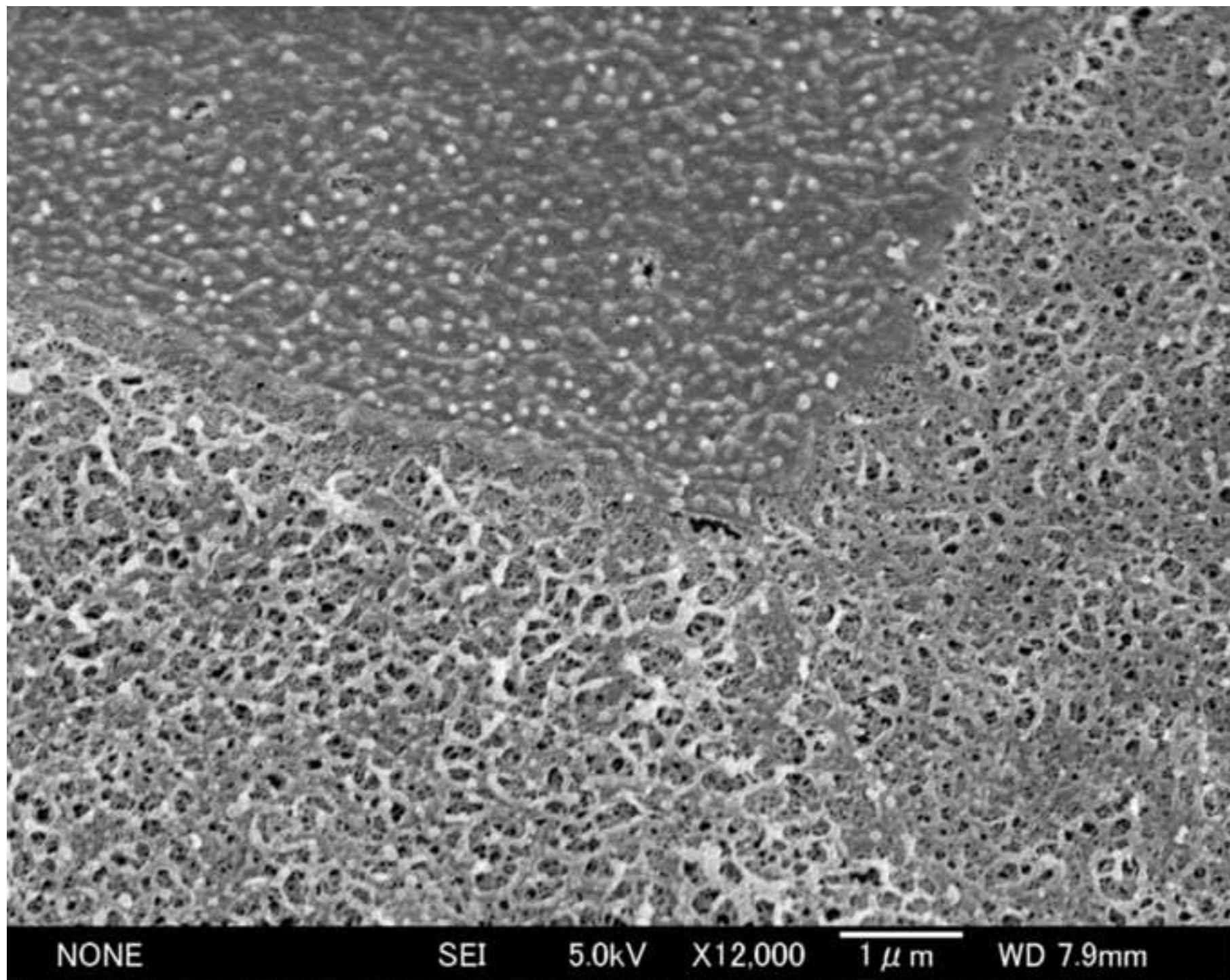


Figure 3G

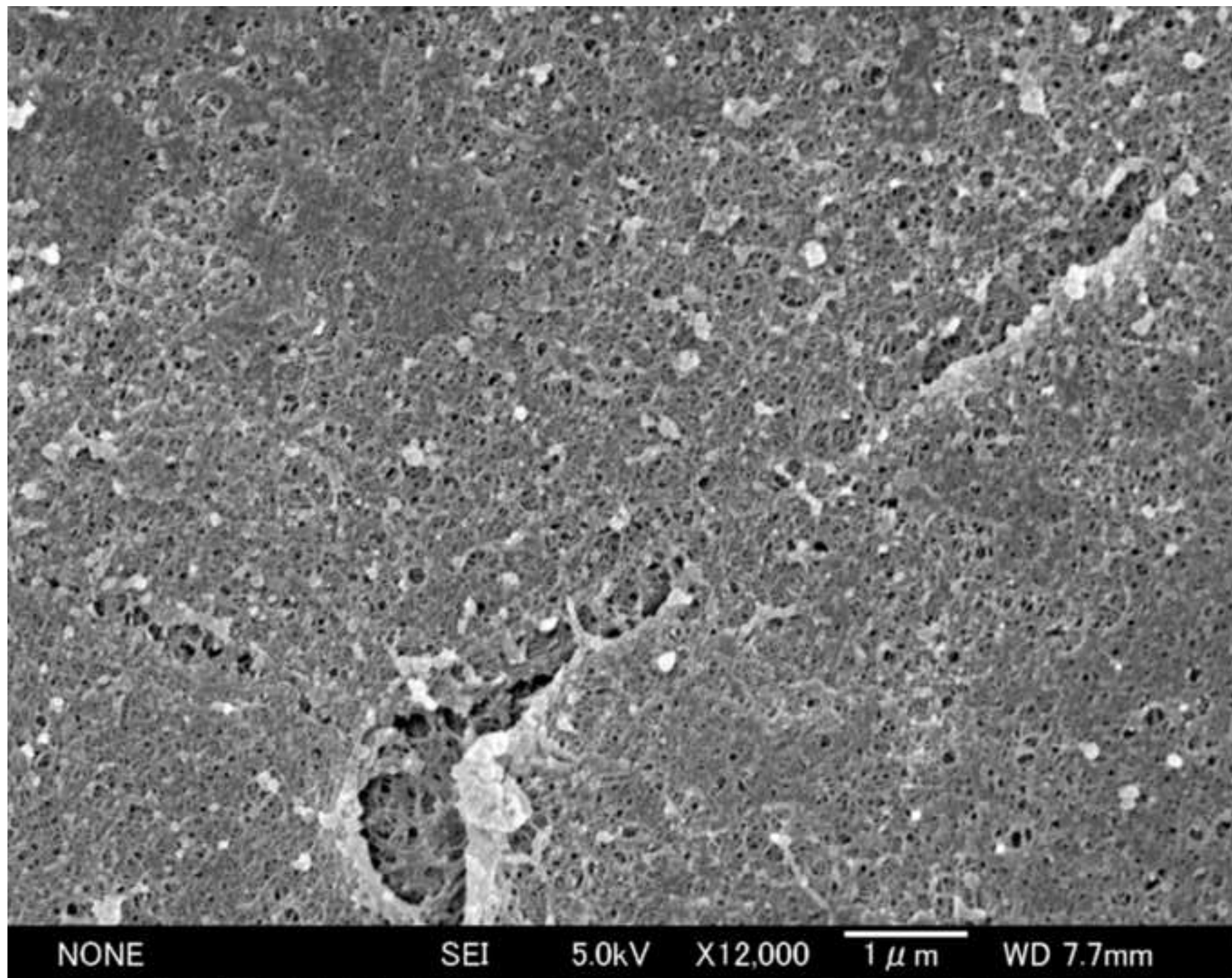


Figure 3H

