A novel approach to evaluate the effect of medicaments used in endodontic regeneration on root canal surface indentation

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Running title: Regeneration medicaments effect on indentation properties of dentine

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Abstract

Objectives*:* To investigate the capability of a novel reference point indentation apparatus to test the indentation properties of root canal surface dentine treated with three intracanal medicaments used in endodontic regeneration.

Materials and Methods: Immature human premolars were selected (n=22). Four specimens were obtained from each root and randomly assigned to three treatment groups and a control group. Each specimen was exposed to one of three treatment pastes (triple antibiotic (TAP), double antibiotic (DAP), or calcium hydroxide [$Ca(OH)_2$] or neutral de-ionized water (control) for one or four weeks. After each time-interval, the indentation properties of the root canal dentine surfaces were measured using a BioDent reference point indenter. Two-way ANOVA and Fisher's Protected Least Significant Differences were used for statistical analyses.

Results: Significant differences in indentation parameters and estimated hardness between all groups at both time points were found. TAP treated dentine had the highest significant indentation parameters, followed by DAP treated dentine, untreated control dentine and $Ca(OH)_2$ treated dentine, respectively. Furthermore, TAP treated dentine had the lowest significant estimated hardness, followed by DAP treated dentine, untreated control dentine and $Ca(OH)_2$ treated dentine, respectively.

Conclusion: BioDent reference point indenter was able to detect significant differences in indentation properties of root canal dentine treated with various medicaments.

Clinical Relevance: The use of a reference point indenter is a promising approach to characterize the indentation properties of root canal surfaces without any surface modification. This might provide an *in vitro* mechanical measurement that is more representative of the actual clinical situation.

Introduction

Endodontic regeneration techniques have been clinically used as a treatment option in patients with immature necrotic teeth [1]. These techniques require disinfection of the root canal with an effective antibacterial regimen for 1-11 weeks [2-4]. The most commonly used medicament during endodontic regeneration is the triple antibiotic paste (TAP), which consists of metronidazole, ciprofloxacin, and minocycline [5, 6]. However, eliminating the minocycline and keeping only metronidazole and ciprofloxacin as a double antibiotic paste (DAP) has been suggested to avoid tooth discoloration [7]. Calcium hydroxide [$Ca(OH)_2$] paste has also been used to disinfect the canal during endodontic regeneration [8].

Traditional work focusing on mechanical properties of radicular dentine after exposure to various root canal irrigants and/or medicaments has utilized Knoop or Vickers indentation techniques to measure microhardness [9-11]. However, there are limitations associated with the use of this regular indentation technique to determine radicular dentine microhardness. Specifically, the difficulty in measuring the precise extensions of the indentations diagonals due to their overlap with dentine tubules and the need for highly polished and flat test samples. Therefore, the radicular dentine tissue that is in direct contact with the intracanal medicament is artificially removed and not tested. Measuring the indentation properties of the intact root canal surface without manipulation would be more representative of the actual clinical situation. Recently, a novel device, the BioDent indenter, has emerged as a tool to measure mechanical properties of bone. Using a technique known as reference point indentation (RPI), this instrument can measure indentation resistance on mineralized tissue surfaces without the need for surface modification [12, 13]. Indeed, recent studies in bone have found a strong correlation between RPI outcomes and nanoindentation [14], tensile mechanical tests [14], or modulus of toughness estimated by three-point bending mechanical tests [15]. Furthermore, clinical studies in bone suggested that some RPI outcomes such as indentation distance increase (IDI) can distinguish between fracture and non-fracture patients [13, 16].

The aim of this study was to investigate the ability of a novel microindentation technique to test the indentation properties of a root canal surface without any surface modification utilizing a reference point indentation approach. It was hypothesized that the three root canal medicaments used in endodontic regeneration techniques have no significant effect on indentation properties of root canal surface dentine using reference point indentation.

Materials and methods

Tooth selection and specimen preparation

Twenty-two intact immature human mandibular premolars were selected after obtaining local IRB approval. The teeth were stored in 0.1% thymol solution at 4 °C and used within six months of extraction. To be included in the study, each immature tooth had to have two-thirds of the root formed and at least a 1-mm-diameter opening at the apical foramen. Additionally, the bucco-lingual diameter of the premolar root had to be 6.8 ± 0.5 mm as measured from the cemento-enamel junction in order to make sure that the root canal surface was wide enough to perform the mechanical test. Each tooth was decoronated horizontally at the cemento-enamel junction, and two 4-mm root dentine cylinders were obtained using a water-cooled low-speed diamond saw (Buehler, Lake Bluff, IL). The pulp tissues were carefully removed using a barbed broach without touching the root canal surface. Then, each cylinder was sectioned into two specimens longitudinally across the maximum diameter of the root canal without touching the root canal surface. Any specimen with a damaged root canal surface was excluded, and the four specimens obtained from that root were replaced. Thus, four specimens were obtained from each root.

Treatment procedure

A TAP was prepared by mixing equal portions of metronidazole, ciprofloxacin, and minocycline (Champs Pharmacy, San Antonio, TX) with de-ionized water (powder/liquid ratio of 3:1). A DAP was prepared by mixing equal portions of metronidazole and ciprofloxacin (Champs Pharmacy) with de-ionized water (powder/liquid ratio of 2.5:1). $Ca(OH)_2$ paste was prepared by mixing $Ca(OH)_2$ powder (Dentonics, Monroe, NC) with de-ionized water (powder/liquid ratio of 2:1). The four specimens obtained from each root were randomly assigned to three treatment groups (TAP, DAP, and $Ca(OH)_2$) and one control group (neutral de-ionized water) in each time point. Thus, each specimen in the three treatment groups had its own control that was obtained from the same root. Each specimen was placed in a 2 mL conical sample cup (Fisher Scientific, Florence, KY) containing 0.15 mL of one of the treatment pastes or neutral de-ionized water. The amount of paste selected was just enough to cover the root canal surface of each specimen. The containers were stored at 37°C for one or four weeks. The two time points were selected to represent the intra-canal application time of medicaments as reported in some cases of endodontic regeneration [4, 17]. For the four-week groups, each specimen was hydrated with 0.07 mL of de-ionized water weekly. After each time interval, 44 specimens from 11 teeth were taken out and rinsed thoroughly with de-ionized water.

Mechanical testing

The indentation properties of the superficial root canal dentine of each specimen were measured using a BioDent H reference point indention apparatus (Active Life Scientific Inc., Santa Barbara, CA). The major components of the indenter are a measurement head unit, a measurement stand that allows for smooth movement of the measurement head unit, and a computer unit. The probe assembly interfaces between the indenter and the hard tissue sample. It consists of a reference probe (a custom sharpened 22 gauge hypodermic needle) and a test probe (a 375 μm diameter rod with a 90° V-shaped end and a 2.5 μm radius spherical tip) that slides through the inside of the reference probe. Each root specimen was mounted on an acrylic block and the probe assembly was passively placed on the center of the root canal surface of the specimens (Fig. 1A). The probe assembly can operate effectively with an angulation of ±7.5° from perpendicular meaning that valid measurements can be obtained even when a perfectly perpendicular orientation is not achieved on the root canal surface [13].

Measurements were performed using the following measurement protocol: indentation force = 5N; indentation frequency = 2Hz; indentations per measurement = 10 cycles. Three measurements, located at least 1 mm apart, were taken and averaged from each dentine specimen. Each probe assembly used in this study was calibrated prior to testing and after every 30 dentine indentations by making three indentations on a poly methyl methacrylate block (Auburn Plastics and Rubbers, Inc, Indianapolis, IN), according to the manufacturer's instructions, to ensure the integrity of the test probe tip. Each root specimen's resistance to indentation was quantified by measuring the following parameters: indentation distance increase (IDI), first cycle indentation distance (ID) and total indentation distance (total ID) (Fig. 1B). These three indentation parameters were selected from various reference point indentation (RPI) outcomes because they were the most common parameters used in previous studies [12, 13, 15, 18, 19].

The hardness values were estimated by dividing the applied force over the estimated conical indentation area created by the test probe using the following equation of cone geometry:

$$\mathrm{Hardness} = \mathrm{P}/(\pi \times \mathrm{r} \times \sqrt{\mathrm{r}^2 \times \mathrm{h}^2})$$

Where P is the constant load applied = 5 Newton; r and h are first cycle ID values of radius and height obtained from the indenter.

Scanning electron microscopy (SEM)

One root specimen was randomly selected from each group after each time point for SEM analysis to confirm the presence of indentations on the intact root canal surfaces and qualitatively observe morphological changes in the surface dentine after various treatments. The selected dentine specimens were sputter coated for 3 minutes with gold/palladium and images were taken with a JEOL 6390LV scanning electron microscope (Peabody, MA).

Statistical analysis

Data were checked for normality using the Shapiro-Wilk test. The effects of group and time on indentation measurements and hardness values were examined using two-way ANOVA followed by Fisher's Protected Least Significant Differences. To satisfy the ANOVA assumptions the analyses were performed using the natural-log transformed hardness data. A 5% level of statistical significance was applied.

Results

For the first cycle ID, the distance indented on the first of the 10 cycles, the group-by-time interaction was not significant ($p=0.63$). After one week, the mean ± SE of first cycle ID of root canal surface treated with TAP, DAP, $Ca(OH)_2$ and untreated control was 70 ± 1, 64 ± 1, 43 ± 2, and 48 ± 1 μm, respectively (Table 1). Additionally, the first cycle ID was significantly lower than the control with $Ca(OH)_2$ treated dentine ($p=0.0081$) while both DAP and TAP treated dentine were significantly higher than the control ($p<0.0001$). Furthermore, first cycle ID values were significantly higher for TAP compared to DAP treated dentine ($p<0.0001$). The first cycle ID values were significantly lower after 4 weeks of treatment compared to one week ($p<0.01$), but significant differences among groups remained.

For the estimated hardness, the group-by-time interaction was not significant ($p=0.86$). After one week, the mean ± SE of estimated hardness of root canal surface treated with TAP, DAP, $Ca(OH)_2$ and untreated control was 0.23 ± 0.01, 0.28 ± 0.02, 0.58 ± 0.03, and 0.49 ± 0.02 GPa, respectively (Table 1). Additionally, the estimated hardness was significantly higher than the control with $Ca(OH)_2$ treated dentine ($p=0.0013$) while both DAP and TAP treated dentine were significantly lower than the control ($p<0.0001$), Furthermore, estimated hardness values were significantly higher for DAP than TAP treated dentine ($p=0.0013$). The estimated hardnesses were significantly higher after 4 weeks of treatment compared to one week ($p=0.0083$), but significant differences among groups remained.

For the IDI, the group-by-time interaction was not significant ($p=0.38$). After one week, the mean ± SE of IDI of root canal surface treated with TAP, DAP, $Ca(OH)_2$ and untreated control was 8.8 ± 0.2, 7.9 ± 0.2, 4.4 ± 0.2, and 5.1 ± 0.1 µm, respectively (Table 2). Additionally, the IDI was significantly lower than the control with $Ca(OH)_2$ treated dentine ($p=0.0227$) while both DAP and TAP treated dentine were significantly higher than the control ($p<0.0001$). Furthermore, IDI values were significantly higher for TAP compared to DAP treated dentine ($p=0.0002$). The IDI values were significantly lower after 4 weeks of treatment compared to one week ($p<0.01$), but significant differences among groups remained.

For the total ID, the group-by-time interaction was not significant ($p=0.34$). After one week, the mean ± SE of total ID of root canal surface treated with TAP, DAP, $Ca(OH)_2$ and untreated control was 74 ± 2, 69 ± 2, 46 ± 1, and 51 ± 1 µm, respectively (Table 2). Additionally, the total ID was significantly lower than the control with $Ca(OH)_2$ treated dentine ($p=0.0042$) while both DAP and TAP treated dentine were significantly higher than the control ($p<0.0001$). Furthermore, total ID values were significantly higher for TAP compared to DAP treated dentine ($p<0.0001$). The total ID values were significantly lower after 4 weeks of treatment compared to one week ($p=0.0015$), but significant differences among groups remained.

SEM images taken at 1100X confirmed the presence of microindentations created during the repetitive loading cycles applied to the untreated root canal specimens (Fig. 2A). SEM images taken at 1500X magnification showed a few areas of exposed collagen matrix among TAP and DAP treated specimens at both time points (Figs 2B and C). Additionally, collagen matrix was easier to detect in images taken at 5000X magnification (Fig 2D). However, no morphological changes were observed among $Ca(OH)_2$ treated specimens and untreated control specimens at either time point (Figs 2E and F).

Discussion

The high density of dentine tubules and/or the less mineralized intertubular dentine matrix of the inter radicular dentine near the pulp may give a unique mechanical characteristic of root canal surface dentine compared to other parts of radicular dentine [20, 21]. Traditional means of assessing mechanical properties of this surface have been through classical micro or nanohardness apparatuses yet these techniques require a high amount of surface polishing that removes a portion, or perhaps all, of the inner radicular dentine. This makes it difficult to fully understand how medicaments of endodontic therapy affect the mechanical properties since the inner radicular dentine is the tissue most likely affected due to its spatial location. Our study aims to overcome this technical challenge by using a novel mechanical testing system that allows direct measurement of the root canal dentine indentation resistance without any surface modification.

Reference point indentation, a relatively new technique, utilizes cyclic micro-indentation to measure resistance to crack initiation/propagation. Previous studies using this device on bone have shown a high and significant correlation between reported indentation properties and modulus of toughness [15], fracture resistance [19], and crack growth toughness [13] determined using more traditional mechanical test approaches. These data provide some level of validation that indentation parameters from RPI correspond with mechanical variables from well-established mechanical testing methods.

Since most published data regarding the mechanical properties of radicular dentine are in the form of hardness values from micro- or nanoindentation, hardness values were estimated in our study through calculation from first cycle ID. The mean hardness values of control dentine from this calculation were 0.49 and 0.52 GPa at both time points. These values are slightly lower than the untreated radicular dentine nanohardness values reported in previous studies, which ranged between 0.55-0.76 GPa [22, 23]. Several factors could account for these differences including the immature nature of the dentine selected in this study, the location of dentine measured, and the technique used. It is well known that the inner root canal dentine surrounding the pulp has lower hardness values compared to underlying primary dentine [24]. The significance trends between hardness values and first cycle ID at both time points were the same in the recent study. Therefore, first cycle ID may be sufficient to rank estimated hardness between various groups in future studies.

Our results showed significantly lower indentation properties (IDI, first cycle ID, total ID) from dentine treated with $Ca(OH)_2$ compared to all other groups. This increase in resistance to indentation is likely the result of reduced collagen on the dentine surface after exposure to $Ca(OH)_2$. A recent study has shown that radicular dentine exposure to $Ca(OH)_2$ caused superficial collagen degradation after only one week [25]. The compromised collagen matrix in the mineralized dentine could lead to a more brittle and less tough, even though harder, substrate. Thus, the indentations created using the reference point indenter on the collagen deficient surface dentine after exposure to $Ca(OH)_2$ might be harder to initiate, yet easier to propagate during cyclic stresses. Although the RPI technique does not allow separation of initiation and propagation of damage, a recent study found that $Ca(OH)_2$ caused a gradual decrease in root resistance to fracture and a gradual increase in microhardness of the same roots with time [26]. Previous work has also shown that $Ca(OH)_2$-treated radicular dentine were more susceptible to fracture [27] .

The indentation properties of both DAP and TAP provided contrasting results to those of $Ca(OH)_2$, with higher indentation properties, indicating that damage was easier to initiate/propagate compared to untreated surfaces. This is likely due to the demineralization effect of these acidic antibiotic pastes [25]. Exposure of the collagen matrix on the surfaces of the root canal dentine treated with TAP and DAP is illustrated in the SEM images (Figs 2B-D). A recent *in vitro* study suggested that both TAP and DAP caused significant and continuous decrease in microhardness of radicular dentine after one and three months, respectively [26]. Additionally, TAP and DAP application for three months caused significant reduction in root resistance to fracture [26]. The ability of minocycline, one of the antibiotics in TAP, to chelate calcium and demineralize dental hard tissues [28, 29] might explain the significantly higher indentation parameters reported among TAP-treated dentine compared to DAP-treated dentine. However, the demineralization effect of TAP and DAP might play a significant role in endodontic regeneration by enhancing the attachment and growth of host stem cells on root canal surfaces through the exposure of embedded collagen fibers and mobilization of various growth factors. Additionally, $Ca(OH)_2$ was also suggested to release some dentine proteins [30] that might be helpful during endodontic regeneration. However, further studies are needed to explore the biological effect of these intracanal medicaments on the outcome of endodontic regeneration.

Our results show a consistent trend in that indentation properties were significantly lower in samples treated for four weeks compared to those from one week. One potential explanation for this is there may have been baseline differences in the indentation properties of radicular dentine of the immature teeth selected for the two time points. Alternatively, the greater indentation resistance after a four-week incubation period might be due to the buffering effect of dentine to balance the high alkalinity of $Ca(OH)_2$ or the high acidity of the antibiotic pastes. Irrespective of the reason, it is important to note that the treatment effects exist, at approximately the same magnitude, at both time points.

Conclusions

The use of a reference point indenter is a promising approach to characterize the indentation properties of root canal surfaces. The null hypothesis stated that intracanal medicaments used in endodontic regeneration techniques have no significant effect on indentation properties of root canal surface dentine using reference point indentation was rejected. The indentation parameters of the root canal surfaces are significantly altered with medicaments commonly used endodontic regeneration. Both antibiotic medications significantly reduced the estimated microhardness of root canal surfaces. However, TAP treated root canal surfaces had significantly less estimated microhardness than DAP treated root canal surfaces. Taking into consideration the unpredictable success rate of endodontic regeneration techniques, our results may have important implications with respect to how surface modifications affect the fracture susceptibility of teeth following endodontic regeneration.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure 1. (A) Illustration of the method for obtaining indentation measurements of root canal surface dentine *in vitro*. 1) Application of the test probe assembly. 2) First-cycle indentation. 3) Last-cycle indentation, which determines the IDI with respect to the first cycle. 4) End of procedure. (B) A typical load-displacement curve obtained from one of the DAP-treated specimens illustrating the way RPI parameters are calculated (only two cycles were included in the curve for clarity).

Figure 2. Representative SEM images from root canal dentine surfaces of specimens treated with various endodontic regeneration medicaments and the control group. (A) Example of an indentation created by BioDent RPI in a four week control specimen (B) Four week TAP-treated specimen with exposed collagen matrix (arrows). (C) Four week DAP-treated specimen with exposed collagen matrix (arrows). (D) Four week TAP-treated specimen with exposed collagen matrix at high magnification (5000X). (E) Four week control specimen with no morphological change. (F) Four week $Ca(OH)_2$-treated specimen with no morphological change.

Figure 1A

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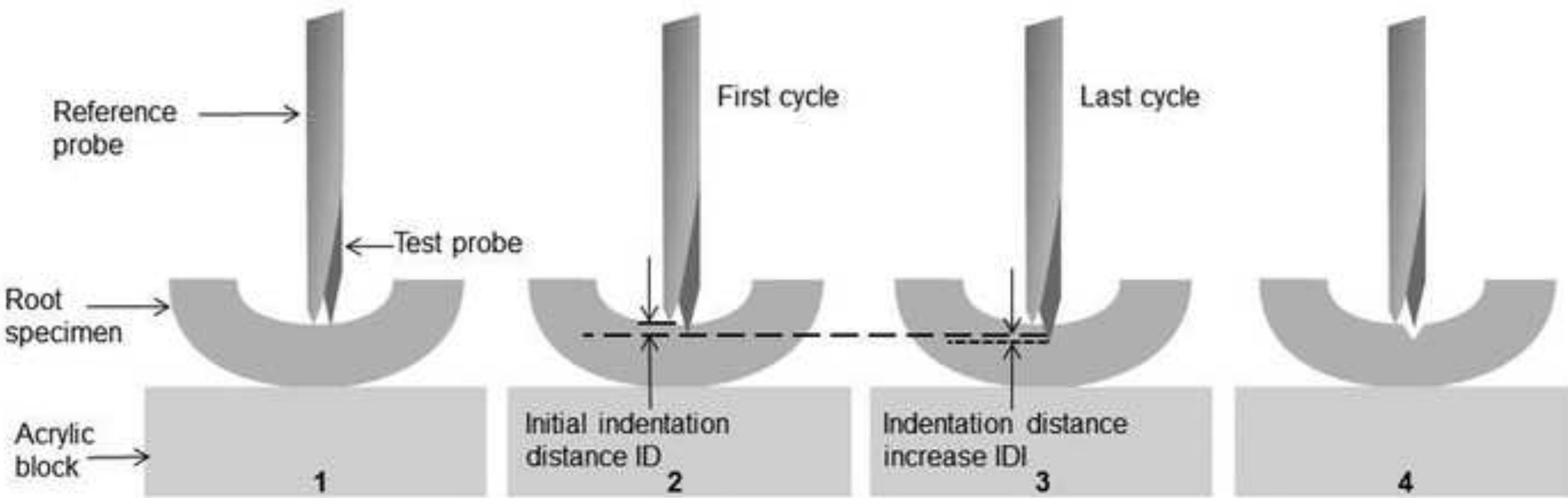

Figure 1B

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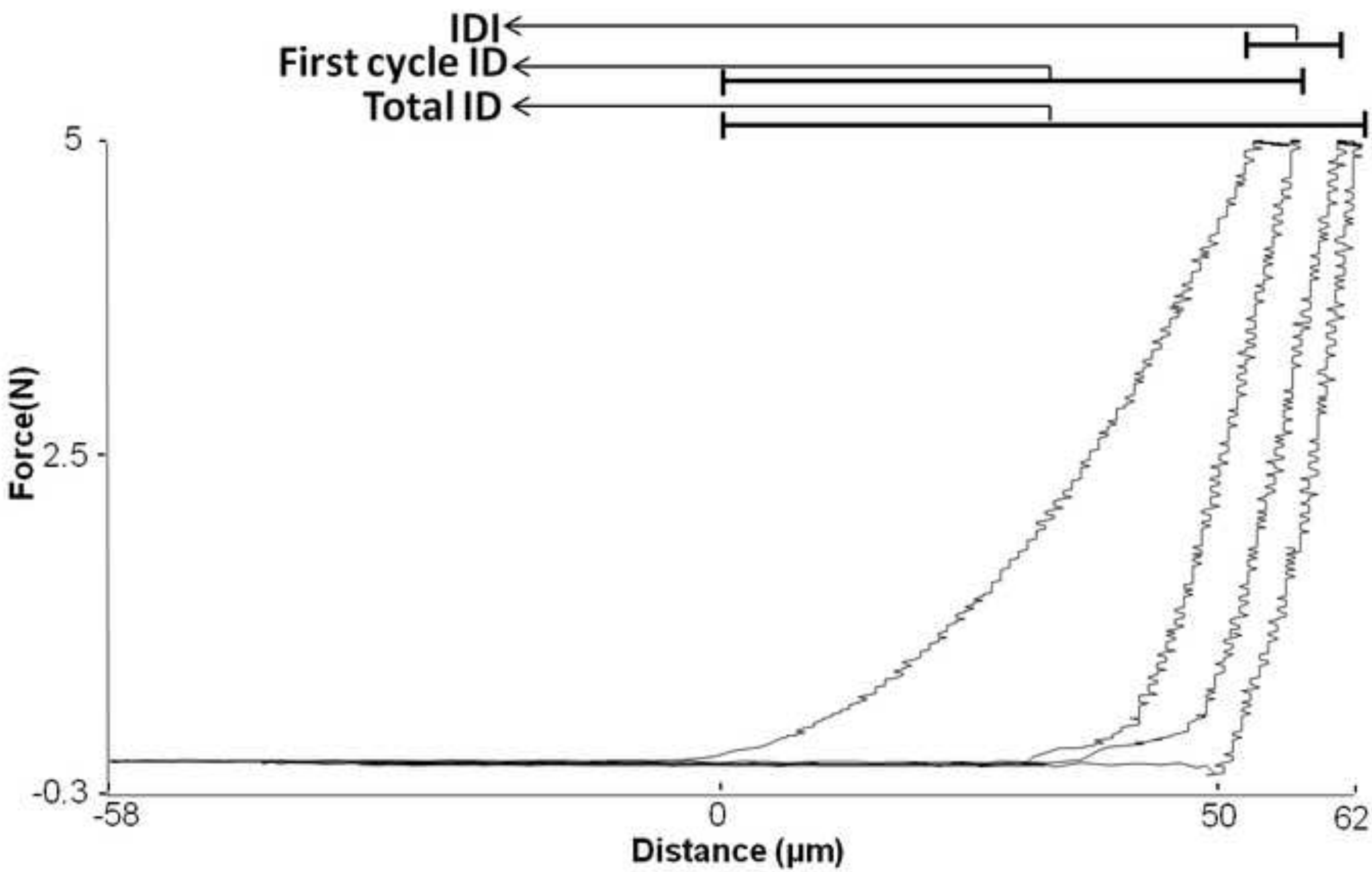

Figure 2A

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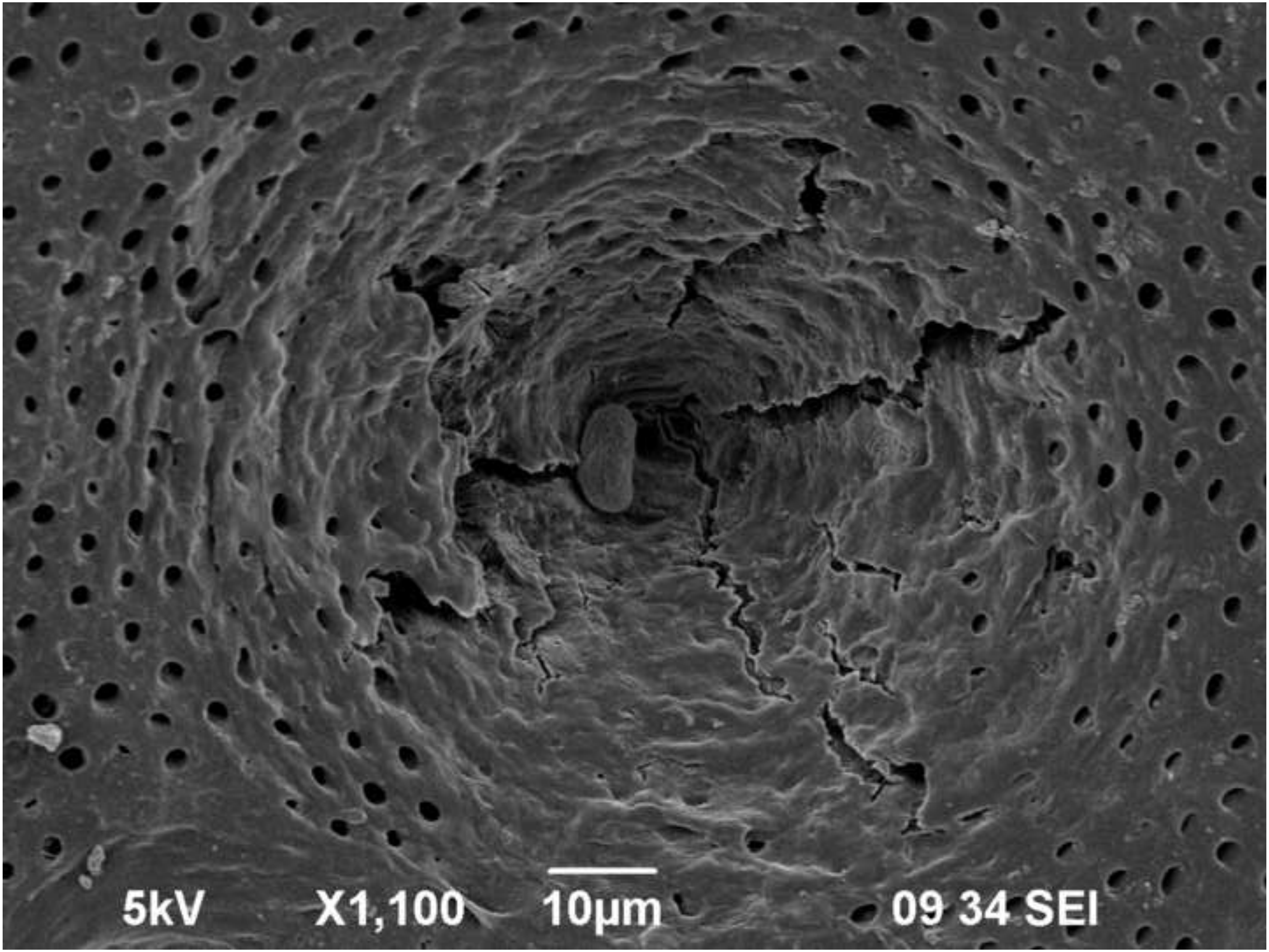

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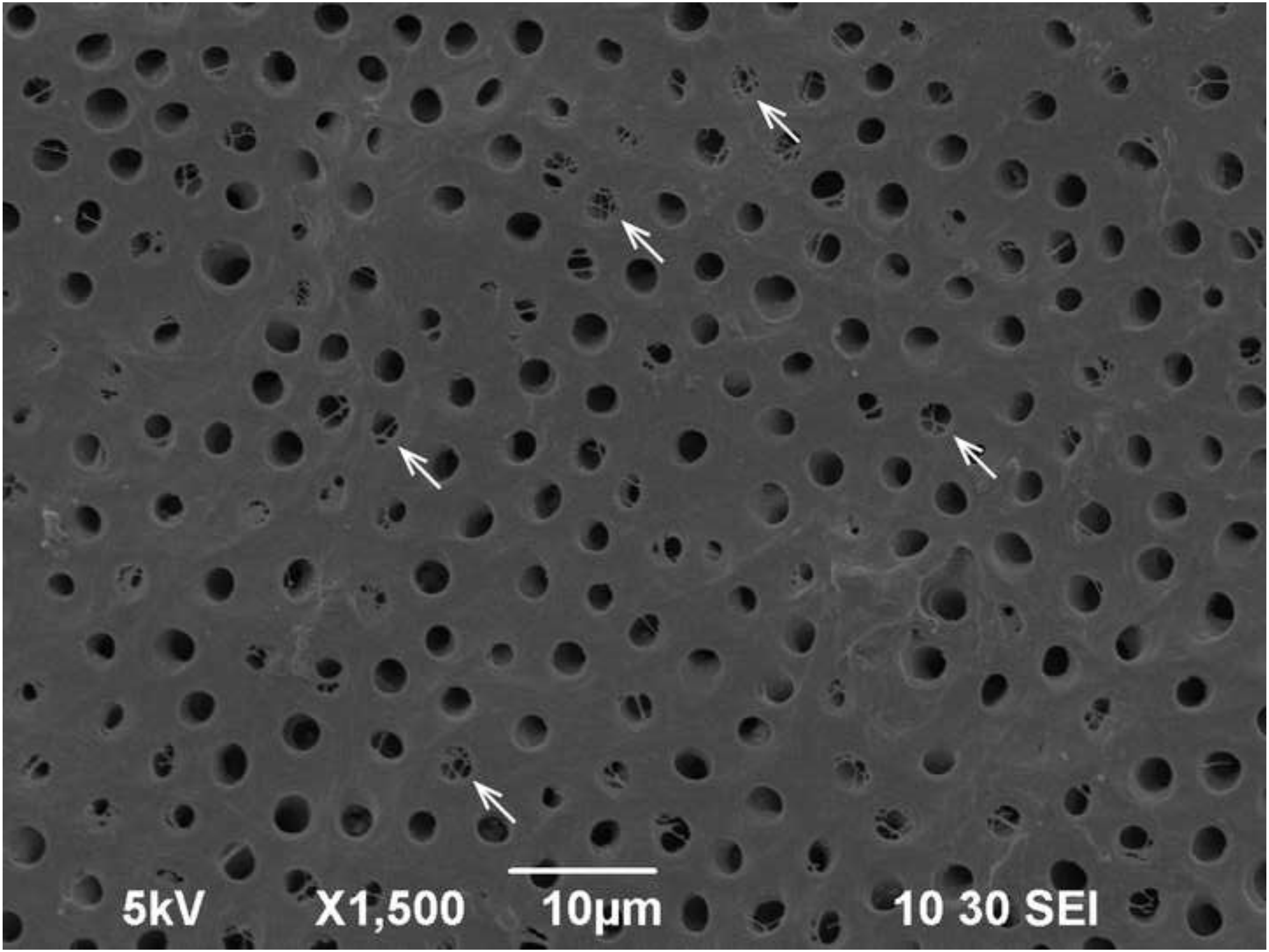

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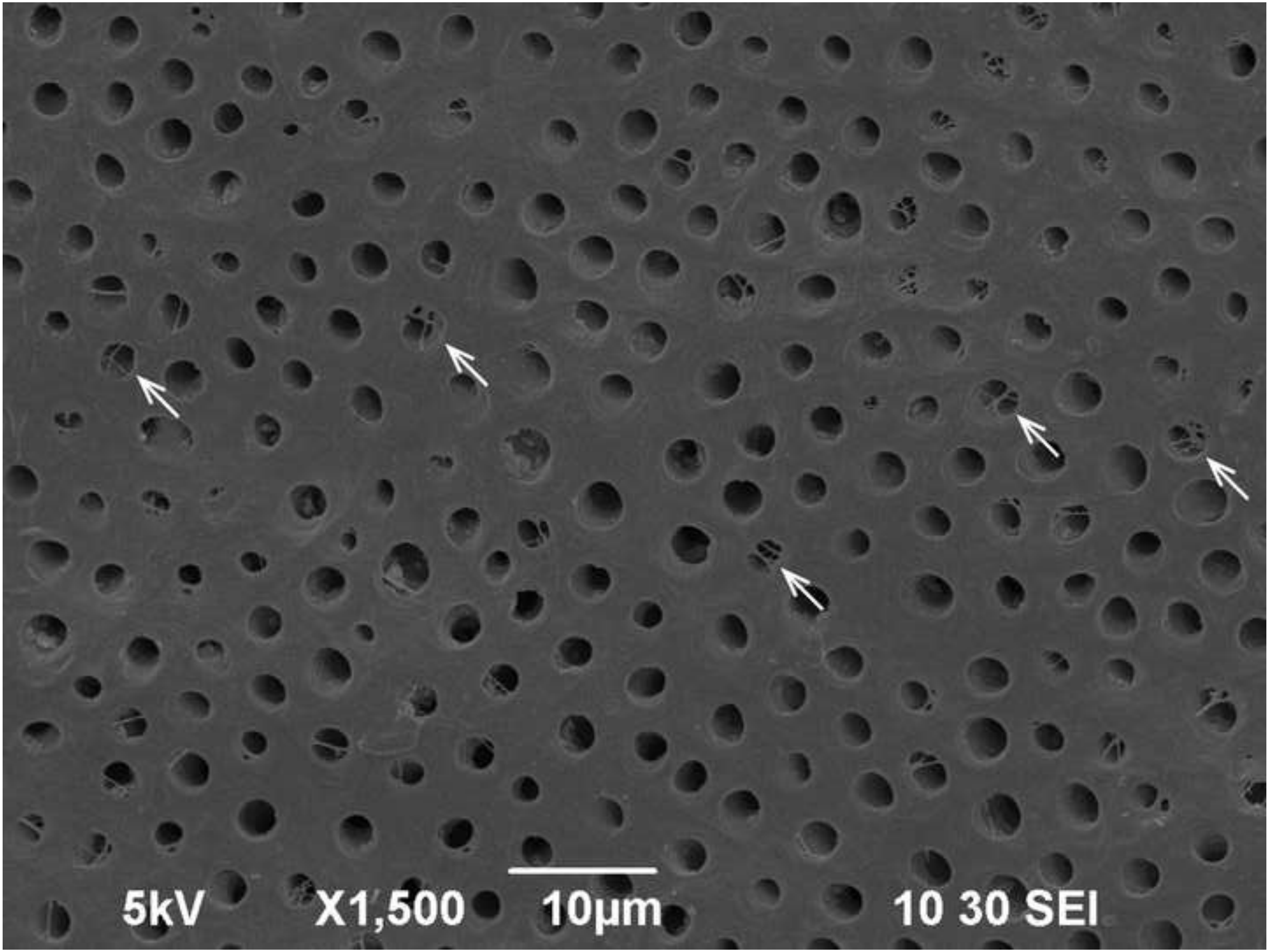

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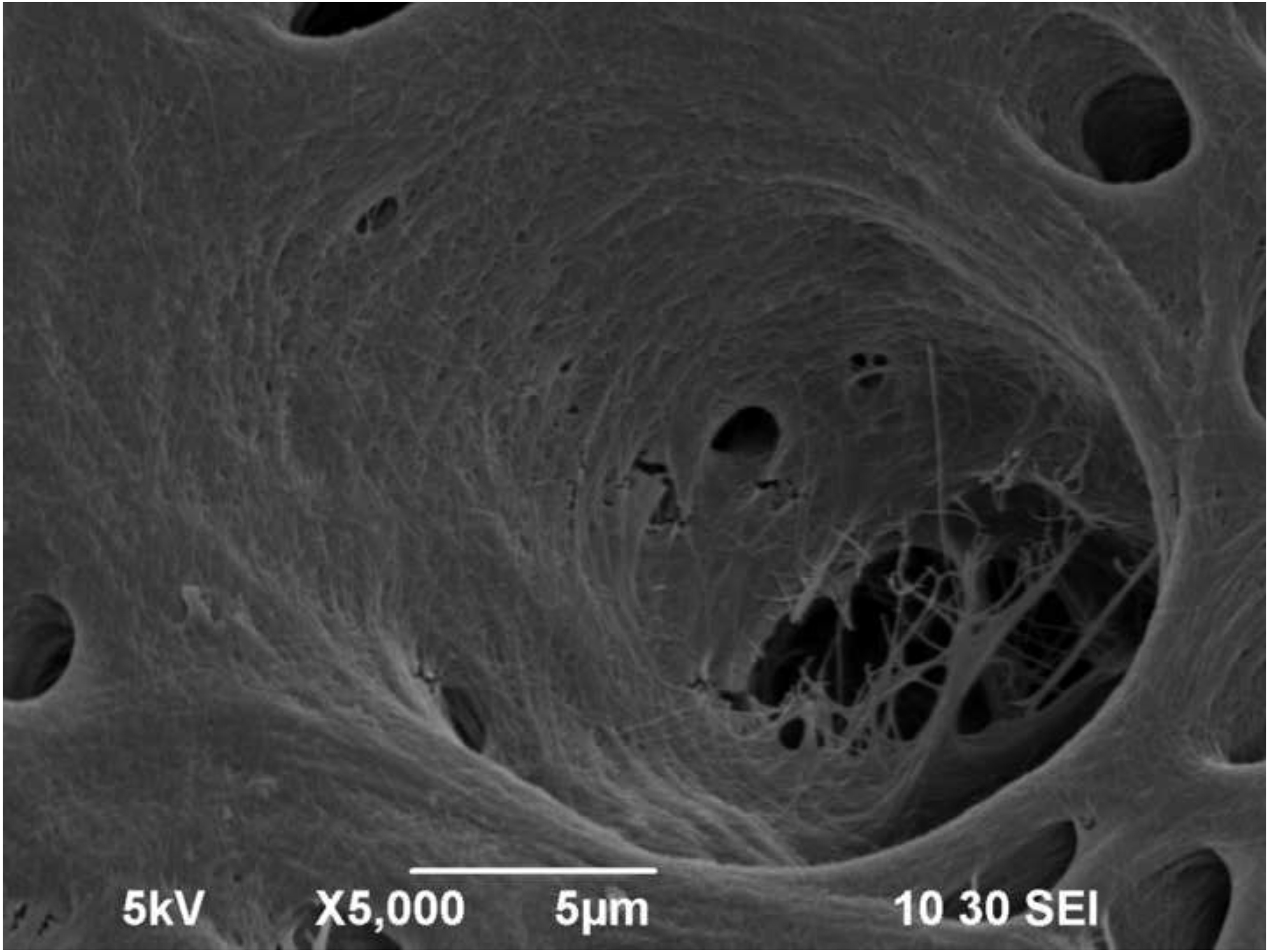

Figure 2E

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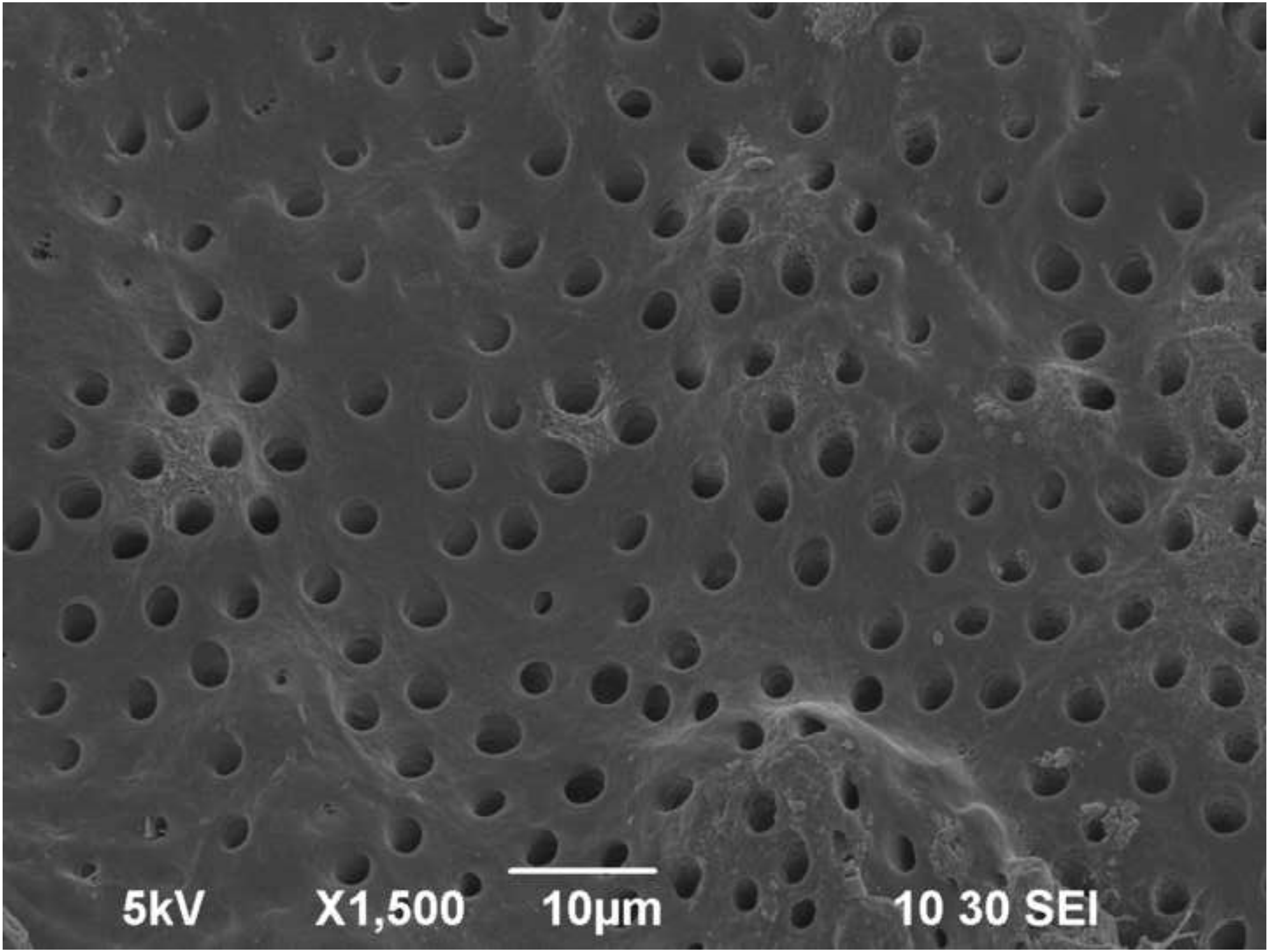

Figure 2F

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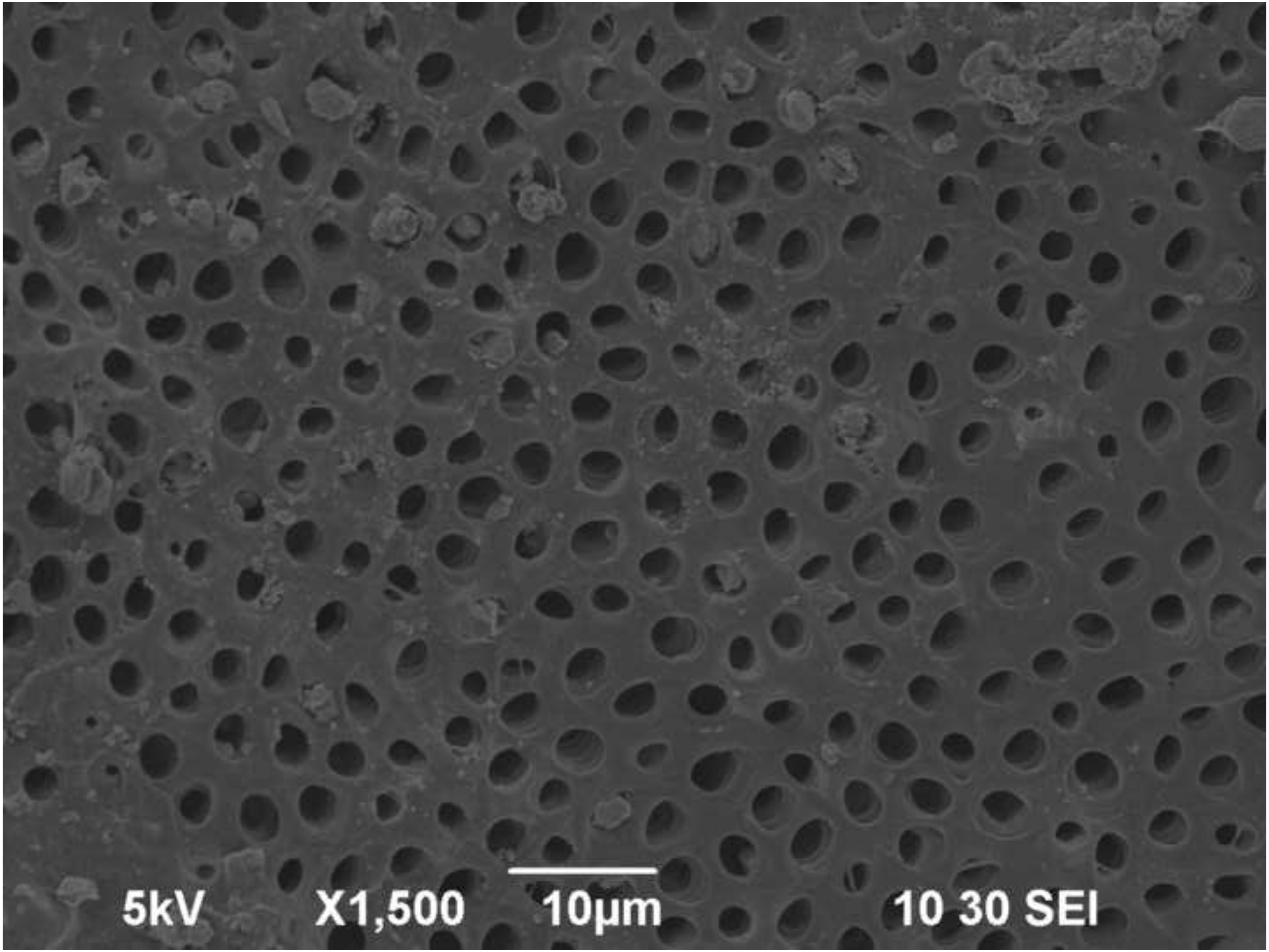

Table 1

Table 1. Mean (SE) of the First cycle ID indentation parameter and calculated Hardness of specimens treated with various endodontic regeneration medicaments and a no treatment control group.

Type of treatment*	First cycle ID (µm)		Hardness (GPa)	
	One-week^	Four-week	One-week^	Four-week
Triple antibiotic paste	70 (1)	66 (1)	0.23 (0.01)	0.26 (0.01)
Double antibiotic paste	64 (1)	61 (1)	0.28 (0.01)	0.31 (0.02)
Untreated control	48 (1)	47 (1)	0.49 (0.02)	0.52 (0.02)
Calcium hydroxide paste	43 (2)	42 (1)	0.58 (0.03)	0.61 (0.03)

* All four treatments were significantly different from each other based on the 2-way ANOVA.
^ One-week and four-week were significantly different from each other based on the 2-way ANOVA.

Table 2

Table 2. Mean (SE) of IDI and Total ID indentation parameters of specimens treated with various endodontic regeneration medicaments and a no treatment control group.

Type of treatment*	IDI (µm)		Total ID (µm)	
	One-week^	Four-week	One-week^	Four-week
Triple antibiotic paste	8.8 (0.2)	8 (0.3)	74 (1)	70 (2)
Double antibiotic paste	7.9 (0.2)	7.5 (0.2)	69 (1)	64 (2)
Untreated control	5.1 (0.1)	4.8 (0.2)	51 (1)	49 (1)
Calcium hydroxide paste	4.4 (0.2)	4.1 (0.1)	46 (2)	45 (1)

* All four treatments were significantly different from each other based on the 2-way ANOVA.
^ One-week and four-week were significantly different from each other based on the 2-way ANOVA.