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Manuscript Draft

Manuscript Number: FSI-D-11-00789R2

Title: Effects of temperature on bone tissue. Histological study of the changes in the bone matrix.

Article Type: Original Research Paper

Keywords: Burned bones, forensic science, forensic anthropology, cremains, thermal

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Abstract: The analysis of burned human remains has given rise to many publications in the literature and has caused great interest among forensic specialists and physical anthropologists due to the difficulty in its analysis and interpretation. The mail goal of this study has been to measure the changes that occur in bone matrix as a consequence of the increased temperature and establishing categories of histological morphology in relation to fire temperature. To this end, a total of 150 bone cylinders from the ilium obtained by bone biopsy. These samples have been obtained from forensic cadavers at controlled temperatures between 100 and 1100˚C in an oven. The samples were fixed in methyl methacrylate and stained with hematoxylin-eosin, Goldner's trichrome and toluidine blue stains. The samples were studied using an optical microscope at 100x. Our study classifies the morphological changes that occur in bone matrix in four stages as a result of the temperature.

Suggested Reviewers:

Title: **Effects of temperature on bone tissue. Histological study of the changes in the bone matrix.**

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Conflicts

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Running title : Burned Human Remains

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Introduction

The analysis of burnt human remains has been of great interest among forensic anthropologists largely due to the difficulty that their recovery, classification, reconstruction, and identification present. The main purpose of this analysis is to determine the best methodology for the preservation and interpretation of burnt bones as a consequence of thermal processes. We include analyses of the distinction between burnt bones with and without flesh, relation between the color and temperature reached by fire, contraction, and macro-microscopic structural changes (1).

In the last decade, the investigations have been focused on the bone surface color and the macroscopic and microscopic morphology of the crystalline structure in a controlled environment (2,3,4,5). However, a review of the anthropological literature reveals that the current methodologies for the burnt human bone analysis is still, at best, confusing. Presently there are no publications centered on the histological structural changes caused by fire.

At a macroscopic level we can observe that during the cremation process, the fire and heat modifies and destroys the bone structure in size, color and shape (6,7). Between 100 and 300 ºC the bone dehydrates, causing a reduction in its size by 1-2 percent of its volume (8). Afterward, between 300 - 600ºC, begins a phase which produces a change to the primary

structural characteristics in the mineralized bone tissue. At a temperature of 600–800ºC, the organic material fully burns and bone structure contraction increases (9). At temperatures higher than 800ºC, the crystals, generated by the increase in temperature, melt into bigger crystals and the bone structure becomes more fragile (10,11). The melting point is approximately at 1630ºC (12).

All of these microscopic changes have led to a wide variety of studies, the majority of which are contradictory, and the few studies on the histological changes have been performed with instruments that are too expensive for some laboratories.

Our research proposes an easily applied method with a simple microscope of which the main purpose is to determine the changes produced in bone matrix as a consequence of the increase in temperature. The study of these changes is necessary due to the important effects that fire and heat produce in the alteration of material evidence and human remains.

Methods.

A total of 165 samples were obtained from forensic cadaver autopsies, with an age range of 26 to 88 years. All individuals had died from unexpected (not necessarily sudden) or violent death. Of the collected samples, 15 were discarded for one or more of the following reasons: time of death exceeded more than 24 hours; insufficient quantity of collected samples; error in sample processing; and evidence of disease that could affect bone metabolism. Of the 150 studied samples, 87 were male and 63 were female.

The samples were obtained by bone biopsy with the use of Bordier's trocar in the left ilium (Fig. 1), no more than 24 hours postmortem. In this step, a transiliac cylinder of 7 mm in diameter and approximately 15 mm in length was obtained, approximately 2 cm from the iliac border (Fig. 1). The samples were burned in a furnace at controlled temperatures ranging from 100°- 1100° C for 20 minutes each. The following lists the number of samples exposed

to each temperature: 13 at 100ºC, 10 at 200ºC, 13 at 300ºC, 15 at 400ºC, 13 at 500ºC, 15 at 600ºC, 13 at 700ºC, 15 at 800ºC, 15 at 900ºC, 15 at 1000ºC, and 13 at 1100ºC.

The bone samples were processed without decalcification using dehydration in 96% alcohol and inclusion within methyl methacrylate. Sections 3 mm thick were prepared using a Reichert microtome. The sections were fixed on microscope slides and stained using hematoxylin-eosin, Goldner's trichrome, and toluidine blue methods.

The samples were analyzed using a Leica DMLB microscope, and the images were captured with a CCD Sony camera, adapted for use with a digitalizing card. Once the bone section images were obtained and digitalized at 100x, they were analyzed.

Results:

At 100°C, micro-fractures appear in the matrix, but the most characteristic sign is the cord-like structure which corresponds to collagen fibers that tend to separate due to the effect of heat and bone micro-fracture (Fig. 2), .

At 200°C The arrangement of the collagen fibers acquire a more rigid and compact tone, leaving this cord-like arrangement from the previous phase to acquire a bar arrangement, conferring a more orderly and parallel arrangement of the fibers (Fig. 3).

At 300°C This phase is very characteristic and the difference lies in that the structure begins to fracture, the cord-like arrangement disappears to give rise to division and partition of the fibers (Fig. 4).

At 400°C, General deformation of the tissue is seen, some of the fibers have been compacted with others giving rise to an irregular surface where they begin to show formations that will later become crystalline structures (Fig. 5).

At 500°C, the crystallization phase begins, the tissue that was previously chaotic becomes crystalline, cubical crystals appear with the onset of this linear macromolecular crystalline polymer phase (Fig. 6).

At 600°C, the cubical crystalline formations disappear and the matrix acquires an irregular crystalloid structure characterized by a compact surface of irregular shapes without a fixed distribution or geometric figures (Fig. 7).

At 700°C, the crystalline structures begin to appear again, this time large, predominantly round structures are characteristic compared to the cubical form (Fig. 8).

At 800°C, the crystalline formations that had begun to form in the previous stage acquire a larger size and even begin to melt with others (Fig 9).

At 900°C, the structure compacts, the large crystals from the previous phase have given way to much smaller rounded crystals (Fig 10).

At 1000°C, the small rounded crystals from the previous phases disappear, giving way to a compact surface made up of microcrystals (Fig. 11).

At 1100°C, the microcrystals tend to group together, leaving the previous compact form to form linear cord-like reticular shapes, with large lacunae appearing between them (Fig.12).

Discussion

In this study, the ilium was chosen to represent the changes that occur in both the cortical and spongy bone of most of the skeleton (16,17). During the burning process, fire affects all the muscles in an exposed body, causing muscle fibers to warm and reduce. The final position of remains is thus affected by muscle and ligament contraction that initiates after approximately 10 minutes at a temperature of 800ºC. This muscle contraction will result in greater exposure of certain anatomical areas and protection of others, not only in relation to the tissue depth but also in relation to how those areas are positioned. These areas of exposure can, in turn, help in obtaining a representative sample to understand varying temperatures reached by the fire (18, 19, 20). The ilium was chosen for this study because it presents both cortical and trabecular bone in an anatomical area not associated with limb attachment and variable heat exposure.

Bone matrix is composed of an inorganic component, calcium phosphate in the form of hydroxyapatite crystals, and an organic component, collagen fibers.

It has been shown that there is an intimate relationship between the organic (protein) and inorganic (mineral) components in the phases that make up the bone matrix. Therefore, incineration of bone affects both the inorganic matrix as well as the organic matrix, though it does it differently in the latter (21).

As can be observed at 100°C, the first significant change is the cord-like separation of the collagen (Fig 2). This is due to a denaturing of the collagen (22) when the structure acquires greater mobility (23,24).

At 200°C, the arrangement of the collagen fibers becomes more rigid and compact because the structure loses its thermal stability (25). From 300°C to 400°C the structure become compact, giving rise to an irregular surface (Figs. 4 and 5). Conversely, linear macromolecular crystalline collagen polymers appear beginning at 500°C (Image 5). This process is due to the disassociation between hydroxyapatite which begins its crystallization and the thermal destruction of collagen (26. 27).

The phenomena of fusion and recrystallization from thermal hydrolysis of the hydroxyapatite and its derivatives is known as sintering and this is responsible for the histological changes in the bone tissue (28). As the temperature increases, the hydroxyapatite has greater crystallinity, in order to form more stable and larger crystals (29). Based on the results and images obtained at 100x, the most important transformation starting at 600°C where the crystallinity of the bone mineral increases, producing a vitreous structure (Image 6). This structure is probably what causes bone remains to gradually lose their consistency at 600°C with the increase in the combustion temperature (30, 31).

However, this tendency becomes inverted at 700-800°C where the crystalline structures become large with predominantly rounded shapes (Image 7) compared to the cubic shapes (Image 8). This is due to recrystallization and conversion of calcium phosphate into tricalcium phosphate, causing greater hardness and mechanical resistance (32).

At temperatures approaching 900ºC, large crystal structures are no longer identifiable. As temperatures reach 900ºC and higher, the bone matrix becomes completely crystalline, yet amorphous and granular in shape. This can be explained by the recrystallization phenomena of the compounds derived from the hydroxyapatite thermal hydrolysis, which results in

retraction, fracture, bone matrix vesicle formation, Haversian canals bursting and, finally, cluster formations (33-35).

Conclusions:

To conclude, we have focused on the histological changes in the collagen polymerization and hydroxyapatite crystallization processes. These temperature-related changes can be classified into four distinct stages (Table 1). This classification can be made by a simple bone biopsy and offers information about the temperatures to which human remains were exposed. Of course, in casework other factors also should be considered, such as duration of elevated temperatures and context.

References

1. G.N. Van Vark. The investigation of human cremated skeletal material by multivariate statistical methods. I. Methodology, Ossa. 1 (1974) 63–95.

2. J.C. Hiller, T.J.U. Thompson, M.P. Evison, A.T. Chamberlain, T.J. Wess. Bone mineral change during experimental heating: an X-ray scattering investigation. Biomaterials. 24 (2003) 5091–5097.

3. T.J.U. Thompson. Heat-induced dimensional changes in bone and their consequences for forensic anthropology, J. Forensic Sci. 5 (2005) 1008–1015.

4. G. Piga , A. Malgosa , T.J.U. Thompson, S. Enzo. A new calibration of XRD technique for the study of archaeological burned human remains. J Archaeol Sci. 35 (2008) 2171-2178.

5. A.M. Christensen. Experiments in the combustibility of the human body. J Forensic Sci. 3 (2002) 466-470.

6. P. Shipman, G. Foster, M. Schoeninger. Burnt bones and teeth: an experimental study of color, morphology, crystal structure and shrinkage, J Archaeol Sci. 11 (1984) 307–325.

7. G.N. Van Vark, J.M Pasveer, A. Arend, W.H.M. Amesz-Voorhoeve, D. Kuizenga, M. Dokládal. Sex diagnosis of the human sternum. Riv di Antrop. 73 (1995) 65-73.

8. P.M. Correia. Fire modification of bone: a review of the literature, in: W.D. Haglund, M.H. Sorg (Eds.), Forensic taphonomy: the postmortem fate of human remains. Boca Raton, CRC Press, New York, 1997. pp. 275–293.

9. B. Herrmann. On Histological Investigations of Cremated Human Remains. Journal of Human Evolution. 6 (1977) 101-103.

10. T.D. Holland. Use of the cranial base in the identification of fire victims, J Forensic Sci. 2 (1989) 458–460.

11. M. Bohnert, T. Rost, S. Pollak. The degree of destruction of human bodies in relation to the duration of fire. Forensic Sci Int. 95 (1998) 11–21.

12. P.M. Correira, O. Beattie. A critical look at methods for recovering, evaluating,and interpreting cremated human remains, in: W.D. Haglund, m.H. Sorg MH, (Eds.), Advances in forensic taphonomy: method theory, and archaeological perspectives. Boca Raton, CRC Press, New York , 2002. pp. 435-450.

13. J.S. Thomsen, E.N. Ebbesen, L. Mosekilde. Static histomorphometry of human iliac crest and vertebral trabecular bone: A comparative study. Bone 30 (2002) 267–274.

14. P.J. Bordier, C.S. Tun. Quantitative histology of metabolic bone disease. Clin Endocrinol Metab. 1 (1972) 197-215.

15. S.G. Leffler, F.S. Chew. CT-guided percutaneous biopsy of sclerotic bone lesions: diagnostic yield and accuracy. Am J Roentgenol. 172 (1999) 1389–1392.

16. S. Vedi, J.E. Compston, A. Webb, J.R. Tighe. Histomorphometric analysis of dynamic parameters of trabecular bone formation in the iliac crest of normal British subjects. Metab Bone Dis Relat Res. 5 (1983) 69–74.

17. S.A. Symes, C.W. Rainwater, E.N. Chapman, D.R. Gipson, A.L. Piper. Patterned thermal destruction of human remains in a forensic setting, in: C.W. Schmidt, Symes (Eds.), The Analysis of Burned Human Remains, Academic Press, London, 2008, pp. 15–54.

18. P.L. Walker, K.P. Miller. Time, temperature, and oxygen availability: an experimental study of the effects of environmental conditions on the color and organic content of cremated bone, Am J Phys Anthropol. 40 (2005) 216–217.

19. E. Bonnucci, G. Graziani. Comparative thermogravimetric, x-ray diffraction, and electron microscope investigations of burnt bone from recent, ancient, and prehistoric age. Atti Della Accademia Nazionale dei Lincei, Sci Fis Matem Natur. 59 (1975) 517–534.

20. J.B. Duffy, J.D. Waterfield, M.F. Skinner. Isolation of tooth pulp cells for sex chromatin studies in experimental dehydrated and cremated remains, Forensic Sci Int. 49 (1991) 127–141.

21. W.J. Landis, K.J. Hodgens, J. Arena, M.J Song, B.F. McEwen. Structural relations between collagen and mineral in bone as determined by high voltage electron microscopic tomography. Microsc Res Tech. 33 (1996) 192-202.

22. E.P. Paschalis, K. Verdelis, S.B. Doty, A.L. Boskey, R. Mendelsohn, M. Yamauchi. Spectroscopic characterization of collagen cross-links in bone. J Bone Miner Res. 16 (2001) 1821-1828.

23. P. Fratzl, H. Gupta, E. Paschalis, P. Roschger. Structure and mechanical quality of the collagen-mineral nano-composite in bone. Journal of Material Chemistry. 14 (2004) 2115–2123.

24. S. Nuzzo, F. Peyrin, P. Cloetens, J. Baruchel, G. Boivin. Quantification of the degree of mineralization of bone in three dimensions using synchrotron radiation microtomography. Med Phys 29 (2002) 2672–2681.

25. P. Roschger, H.S. Gupta, A. Berzlanovich. Constant mineralization density distribution in cancellous human bone. Bone. 32 (2003) 316–323.

26. D. Ruffoni, P. Fratzl, P. Roschger, K. Klaushofer, R. Weinkamer. The bone mineralization density distribution as a fingerprint of the mineralization process. Bone. 40 (2007) 1308–1319.

27. H.S. Gupta, J. Seto, W. Wagermaier, P. Zaslansky, P. Boesecke, P. Fratzl . Cooperative deformation of mineral and collagen in bone at the nanoscale. Proc Natl Acad Sci. 103 (2006) 17741-17746.

28. W.M. Bass. R.J. Jantz. Cremation weights in East Tennessee. J. Forensic Sci. 49 (2004) 1-4.

29. K.A. Gross, C.C. Berndt. Biomedical application of apatites, in: M.J. Kohn, J. Rakovan, J.M. Hughes (Eds.), Phosphates: Geochemical, Geobiological and Material Importance, Reviews in Mineralogy and Geochemistry, Washington, DC, 2002, pp. 631–672.

30. W.A Merz, R.K. Schenk. A quantitative histological study on bone formation in human cancellous bone. Acta Anat. 76 (1970) 1-15.

31. B. Herrmann. On Histological Investigations of Cremated Human Remains. J Hum Evol. 6 (1977) 101-103.

32. E.P. Paschalis, K. Verdelis, S.B. Doty, A.L. Boskey, R Mendelsohn, M. Yamauchi. Spectroscopic characterization of collagen cross-links in bone. J Bone Miner Res. 16 (2001) 1821-1828

33. T.J. Thompson. Recent advances in the study of burned bone and their implications for forensic anthropology. Forensic Sci Int. 146 (2004) S203-205.

34. G. Grupe, S. Hummel. Trace element studies on experimentally cremated bone. I. Alteration of the chemical composition at high temperatures, J. Archaeol. Sci. 18 (1991) 177–186.

35. J.L Holden, P.P Phakey, J.G. Clement. Scanning electron microscope observations of heat-treated human bone, Forensic Sci. Int. 74 (1995) 29–45.

Figure 1: Location of bone biopsy taken from the ilium.
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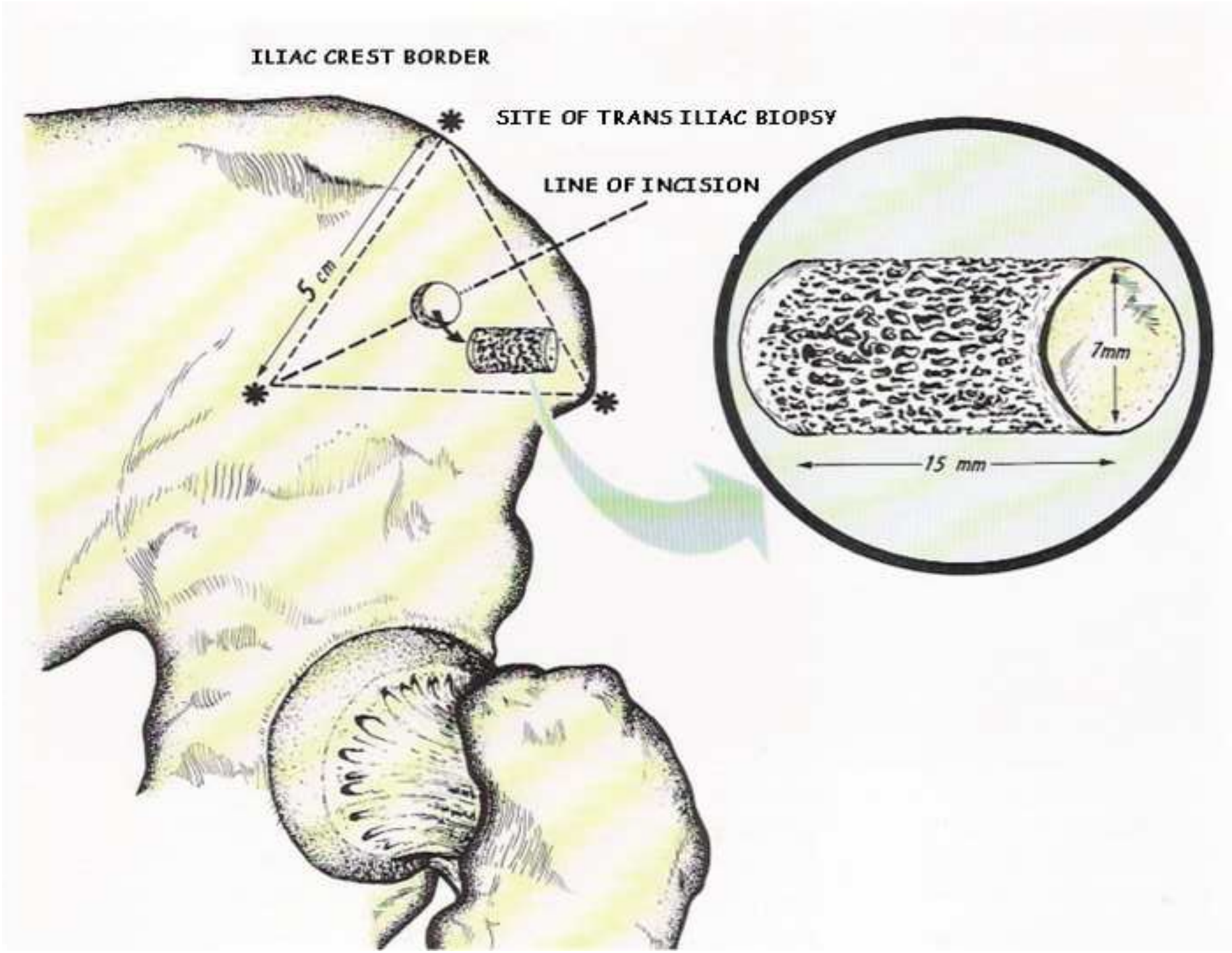

Figure 2: Deposition of collagen fibers in the bone matrix. 100º
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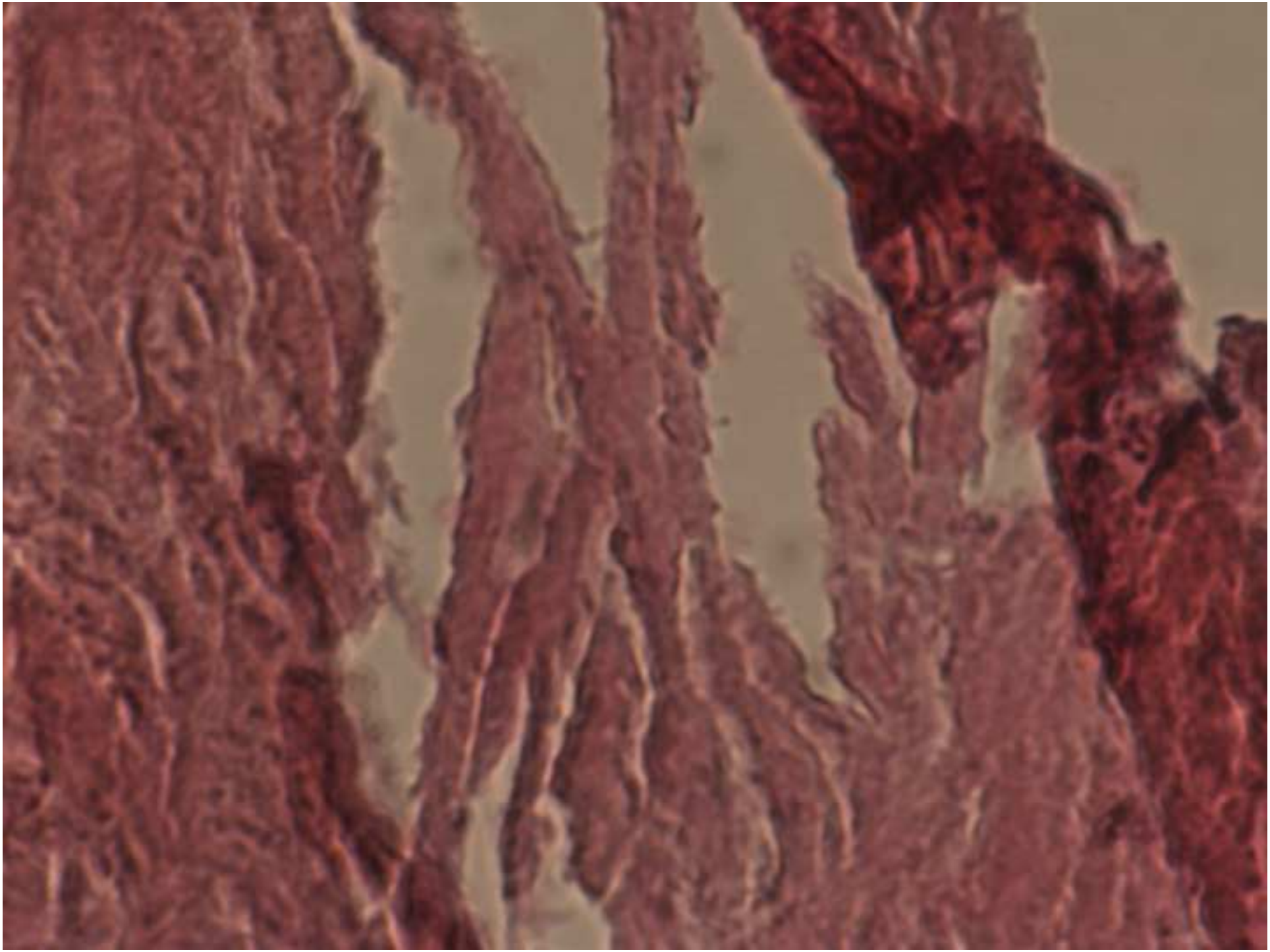

Figure 3. Deposition in bars. at 200ºC
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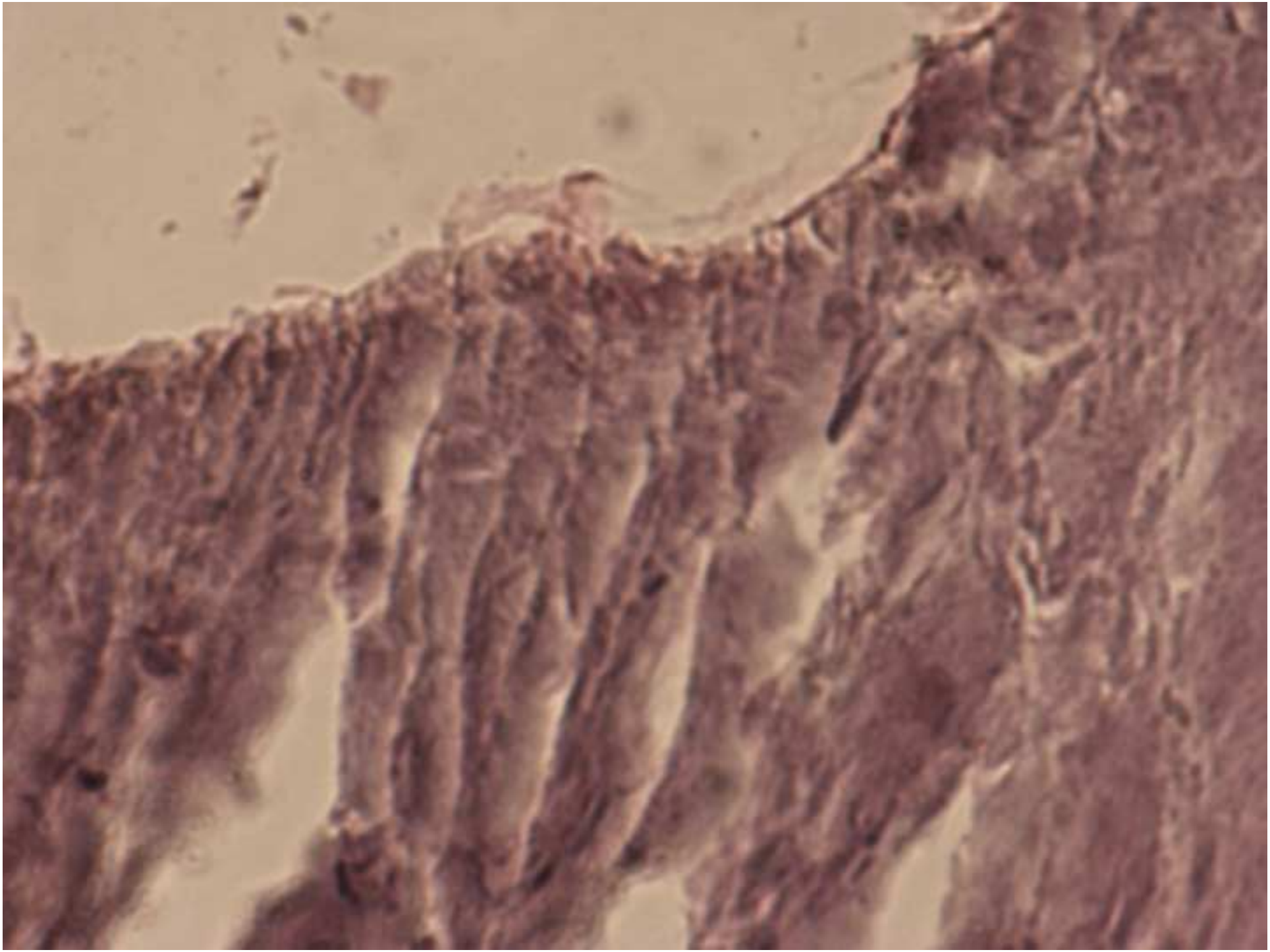

Figure 4: Fracture of the fibers at 300ºC
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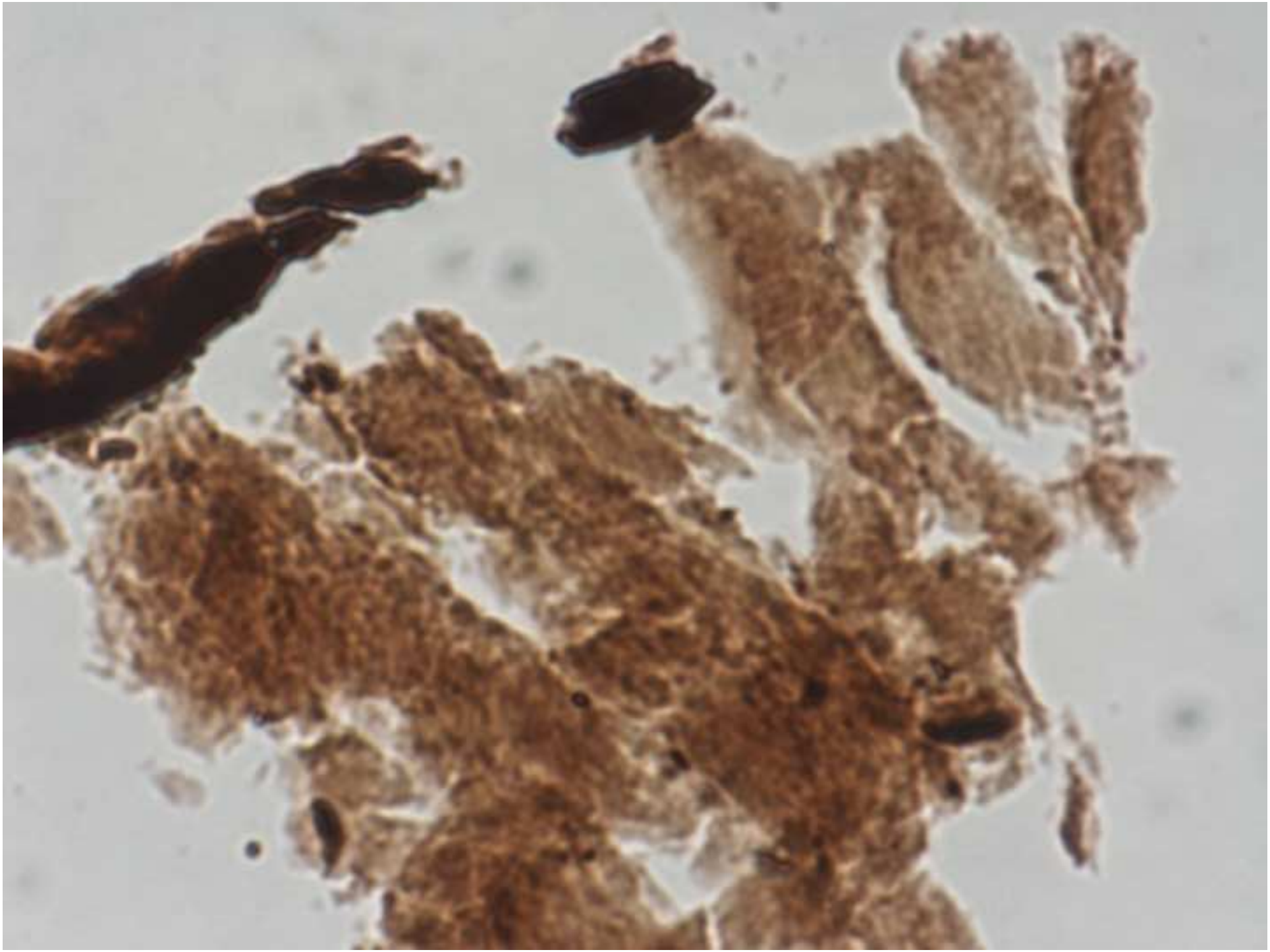

Figure 5: Compacting of the fibers at 400ºC
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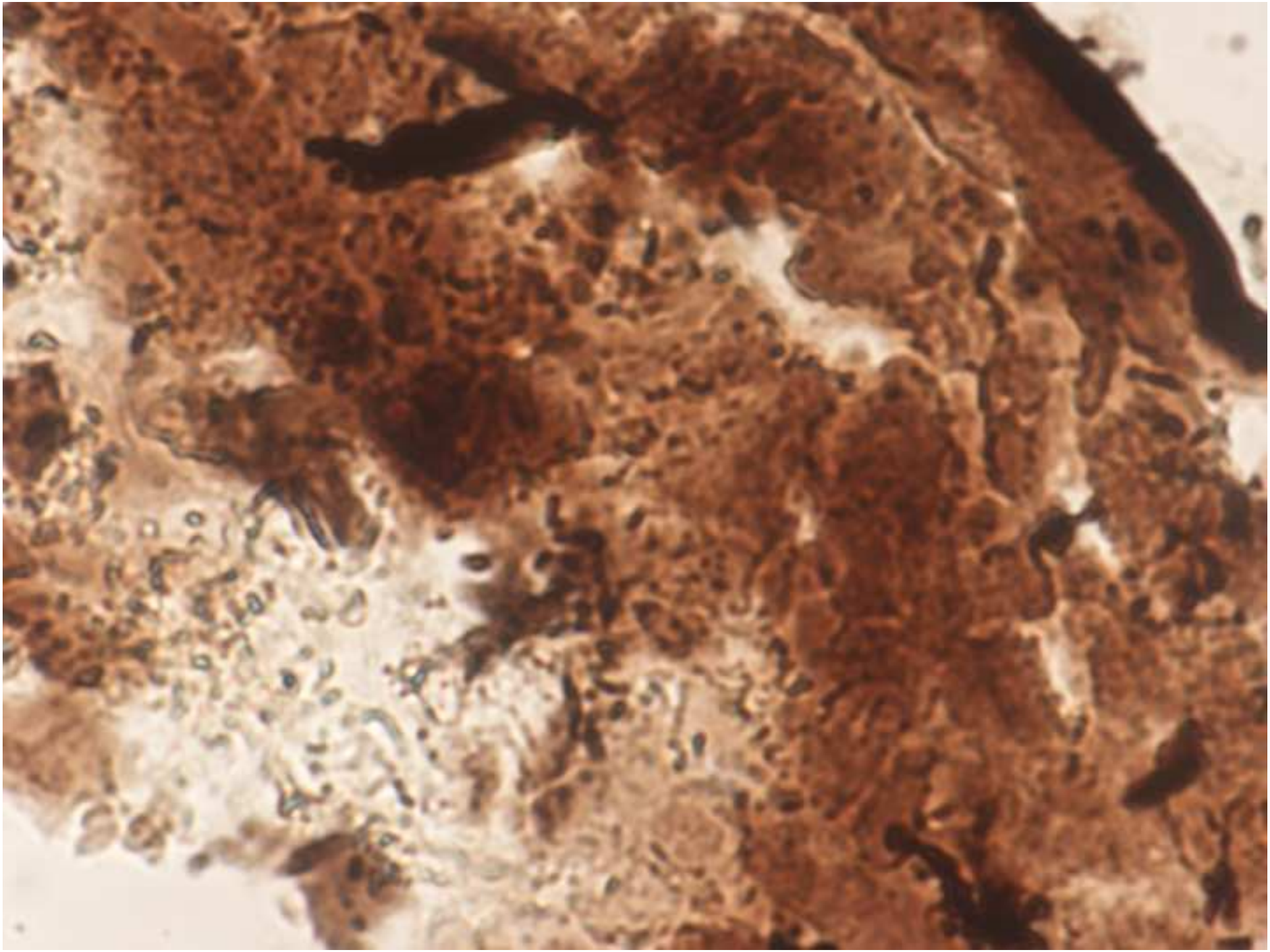

Figure 6. Crystalline structure at 500ºC
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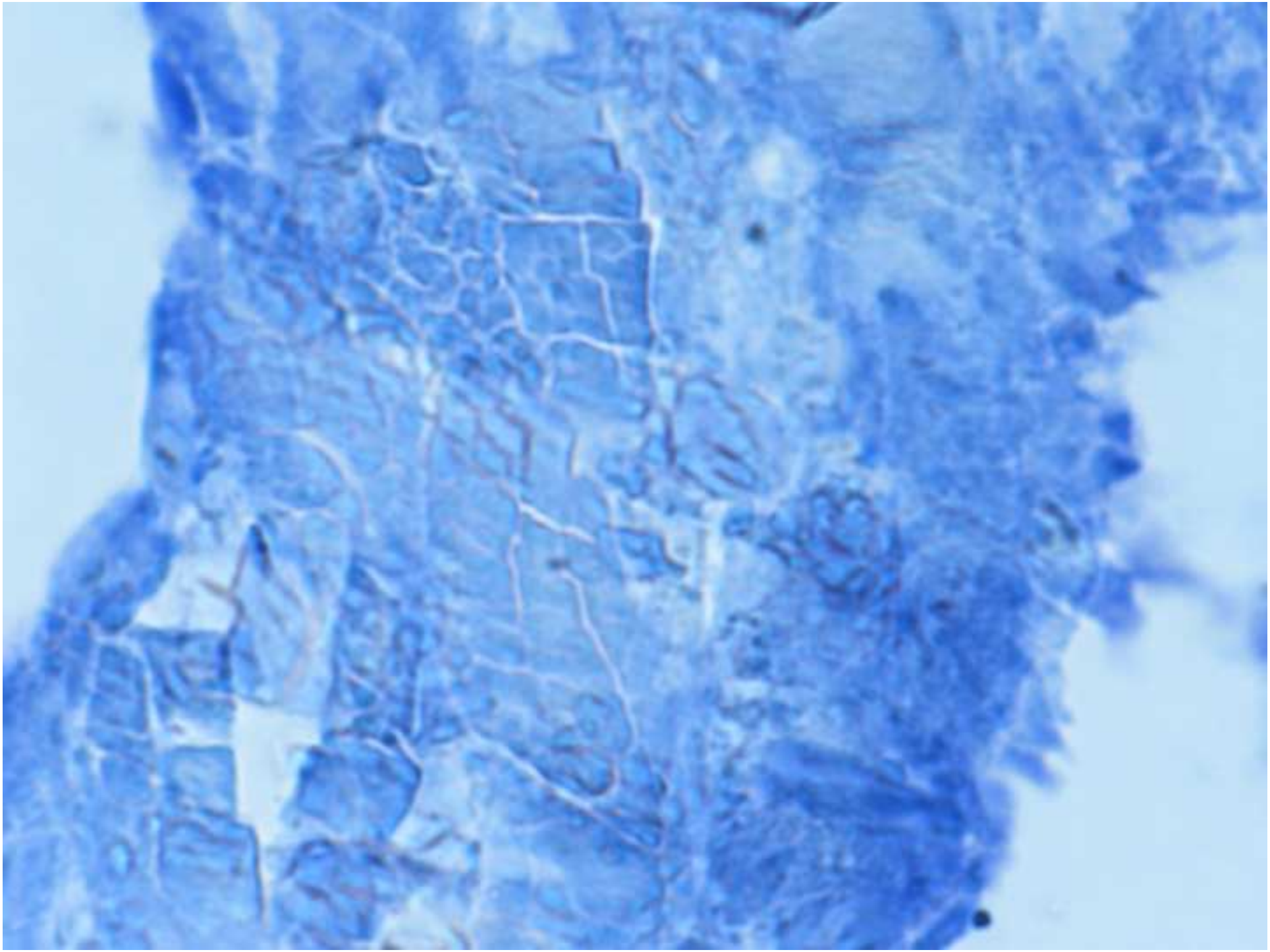

Figure 7. Compact crystalline matrix at 600ºC
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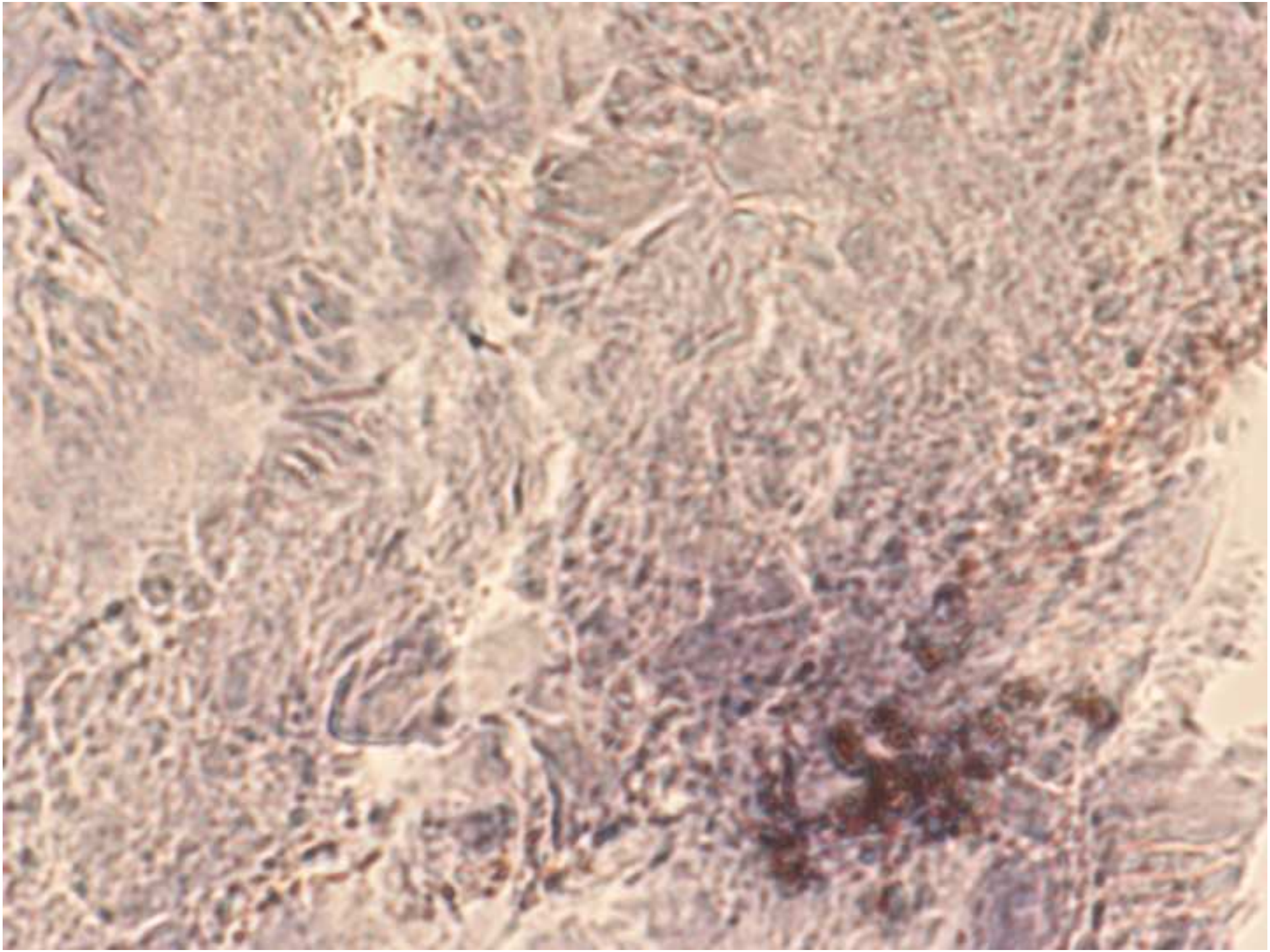

Figure 8. Rounded crystals at 700ºC
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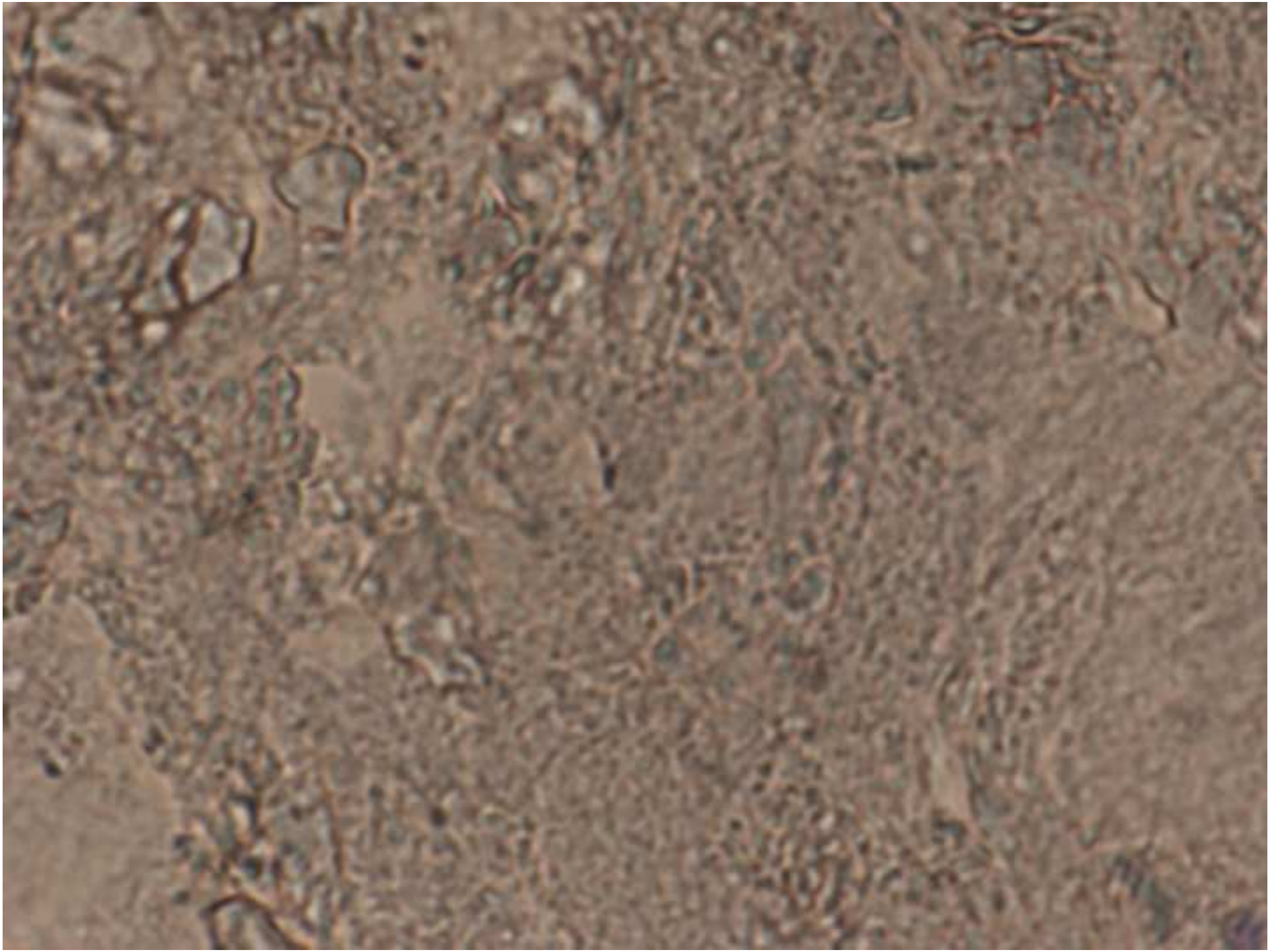

Figure 9: Large crystalline formations at 800ºC
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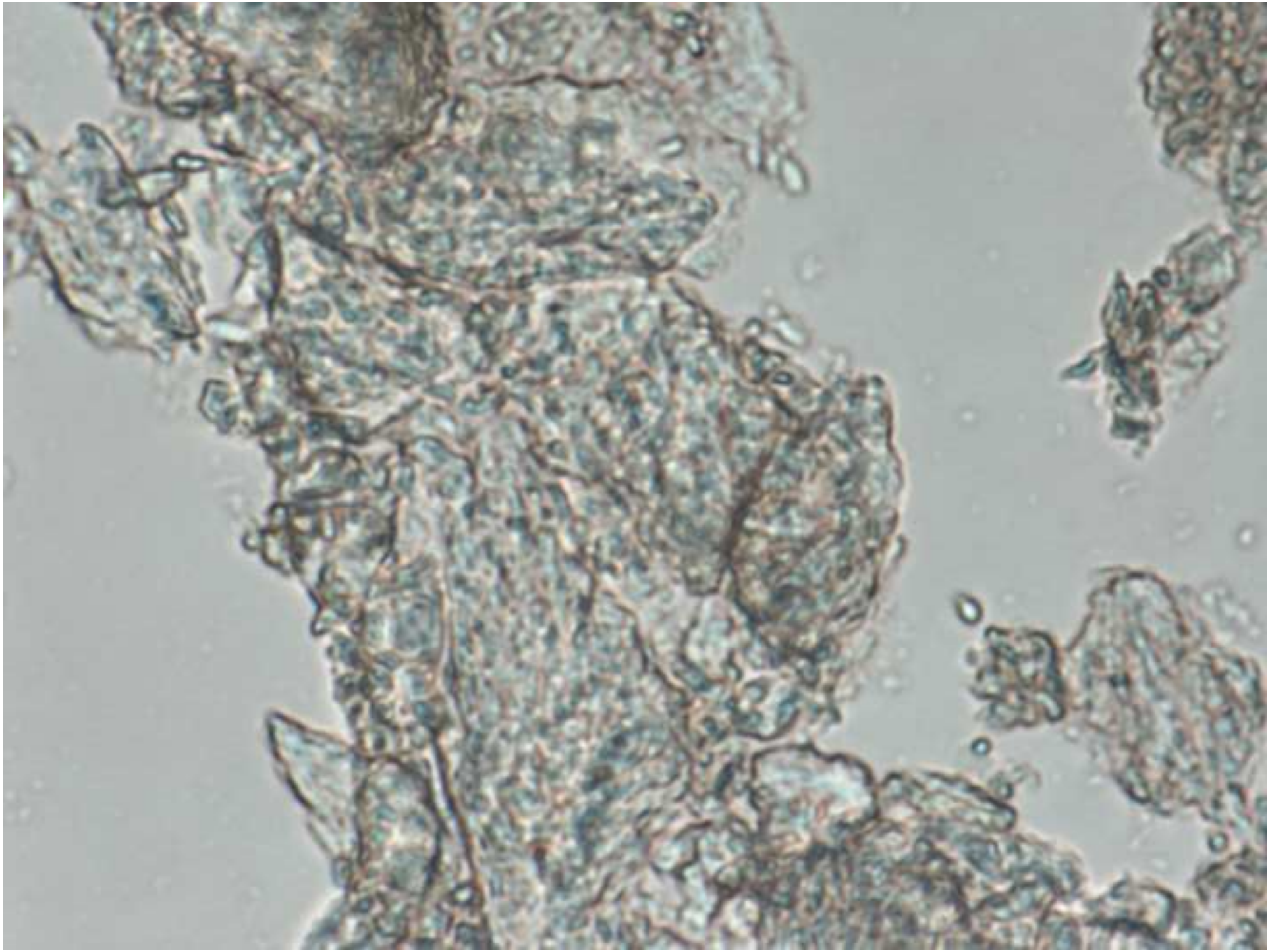

Figure 10: Round-shaped crystals at 900ºC
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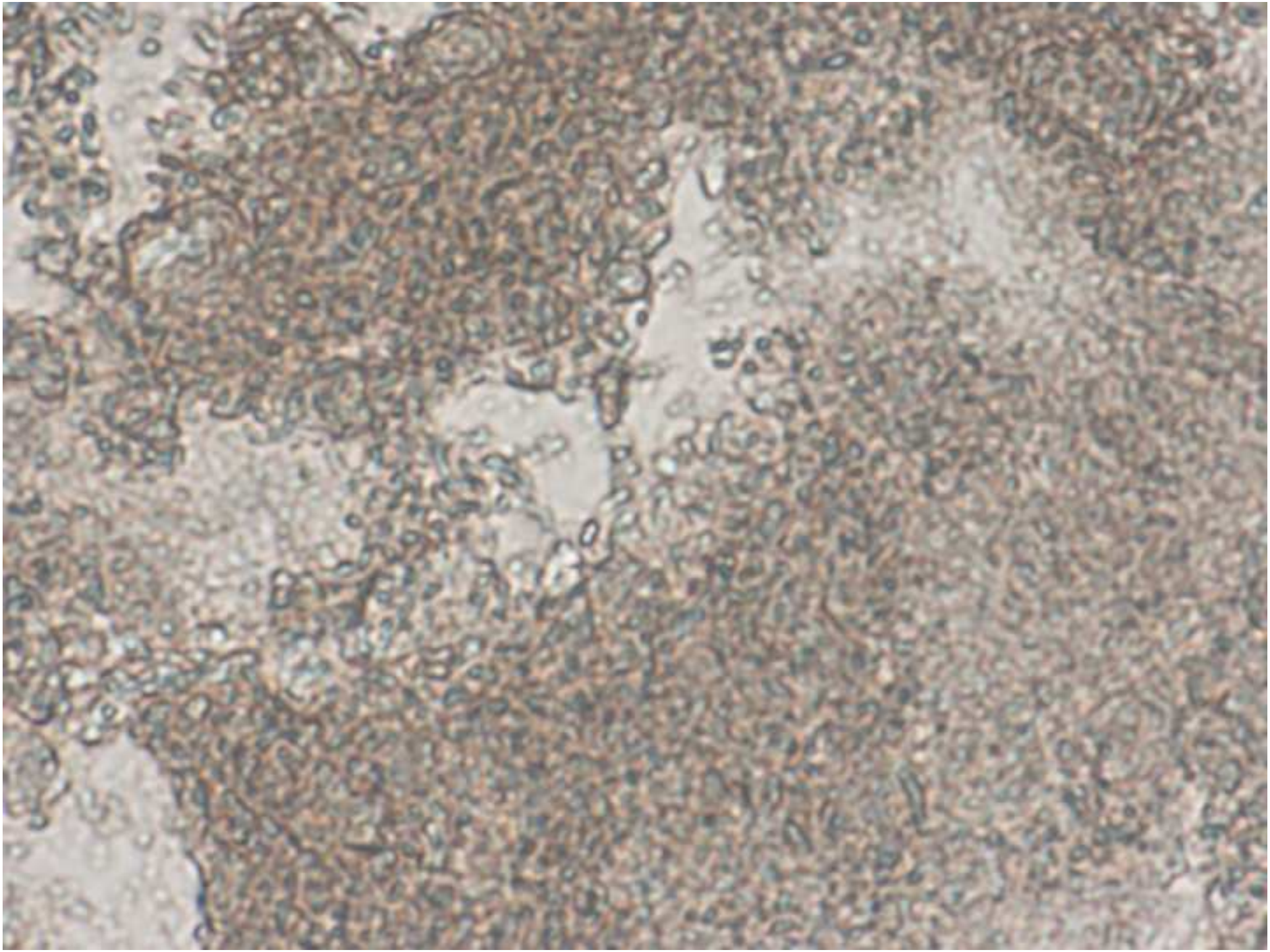

Figure 11: Microcrystals at 1000ºC
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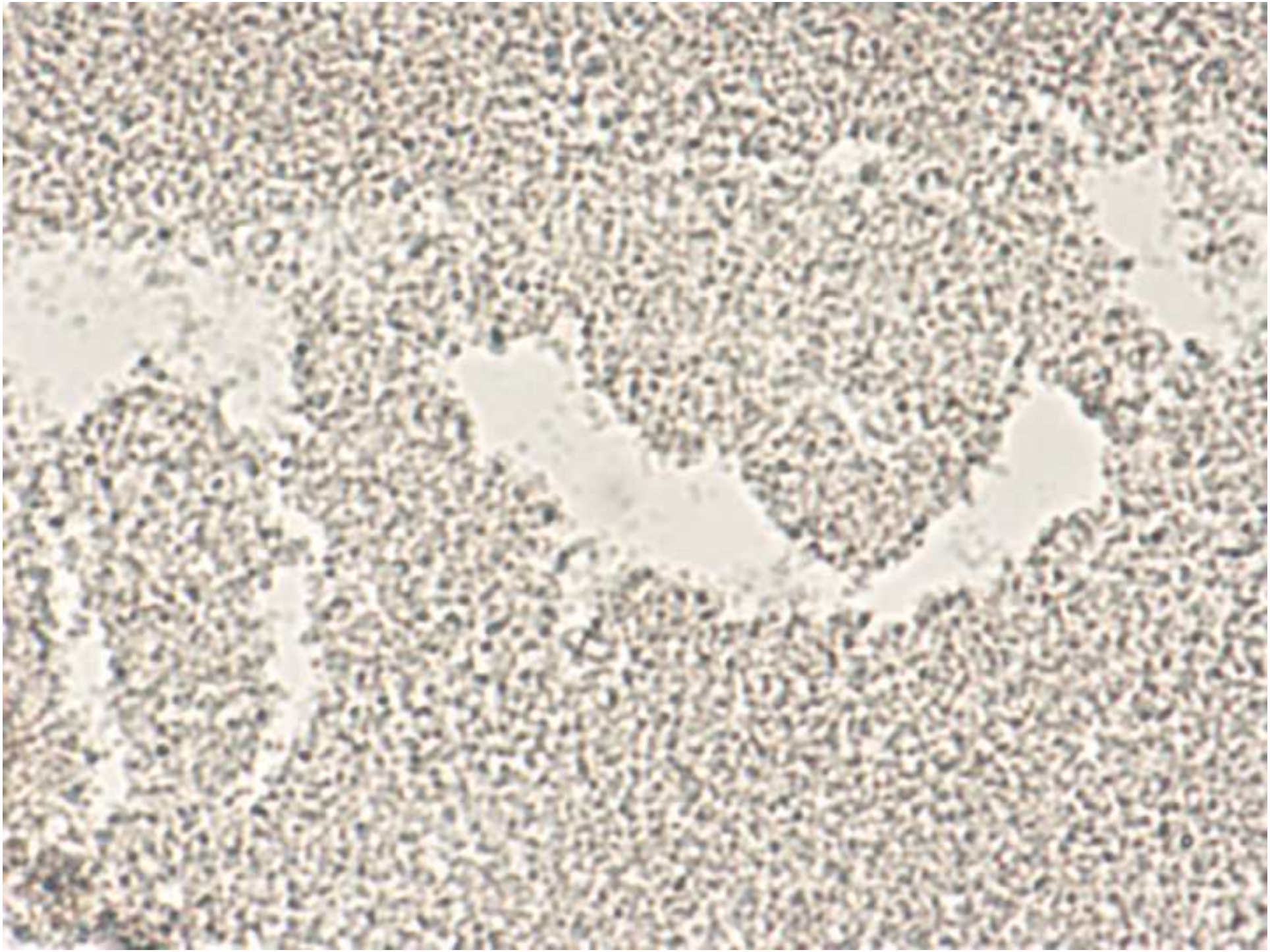

Figure 12: Microcrystals grouped together in cord-like shape.
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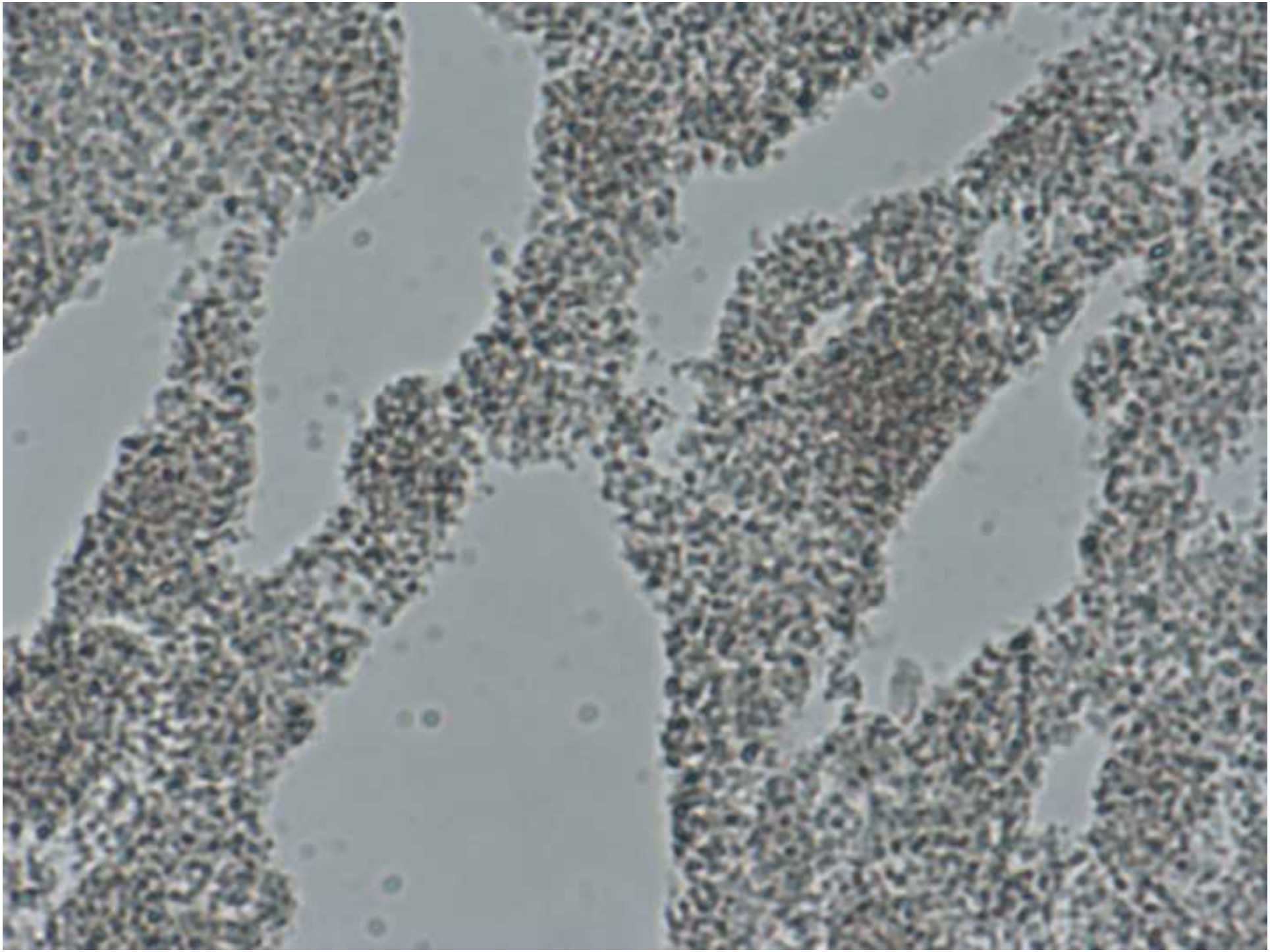

Table 1. Classification by stages of changes

Temperatures	**Histological Changes**
From 100 to 300˚C	Collagen deformation.
From 400 to 600˚C	Vitreous crystalline formations. Crystalline polymers.
From 700 to 800˚C	Presence of rounded and cubical crystals, loss of homogeneity.
Greater than 900˚C	Granular surfaces

***Revision Notes**

- The typos have been corrected.

- Graph 1 provides detailed information about the method of obtaining bone biopsy

- The evaluation of bone diagnostic and peri-implantary bone apposition requires laboratory techniques that allow the evaluation of hard tissues without needing to decalcify them, because the burned bone is very fragile, and when cut with a microtome easily breaks. The relevance of this research it is the classification, the discussion of my point of view it is complete and do not need more explication because is not necessary explain bone histomorphometry in this paper.

Here you are the:

Captions (legends):

Figure 1: Location of bone biopsy taken from the ilium.
Figure 2: Deposition of collagen fibers in the bone matrix at 100ºC.
Figure 3. Deposition in bars at 200ºC.
Figure 4: Fracture of the fibers at 300ºC.
Figure 5: Compacting of the fibers at 400ºC.
Figure 6: Crystalline structure at 500ºC.
Figure 7: Compact crystalline matrix at 600ºC.
Figure 8: Rounded crystals at 700ºC.
Figure 9: Large crystalline formations at 800ºC.
Figure 10: Round-shaped crystals at 900ºC.
Figure 11: Microcrystals at 1000ºC.
Figure 12: Microcrystals grouped together in cord-like shape at 1100ºC.