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Synovial Fluid in joint Patholgy by the Method of Scanning Electron Microscopy

R.Z. Alekseev[1,2], A.S. Golderova[2], C.N. Mamaeva[2], V.A. Platonova[2], O.G. Markova[3]

[1]Yakutsk Scientific Center of Complex Medical Problems, Russia.
[2]North-Eastern Federal University named after M.K. Ammosov, Russia.
[3]Yakutsk Medical Clinic "Lotos", Russia.

ABSTRACT
Despite widespread microcrystalline arthritis, its diagnosis and treatment is still difficulty. The aim of the study was to characterize the crystals of synovial fluid in various pathologies of the knee joint by the method of a scanning electron microscope with energy dispersive analysis of spectrum elements using the "INCA Energy" system. Dry smears of synovial fluid obtained during puncture of the knee joint of patients with various forms of arthritis were analysed. In gout, a large number of microcrystals are observed in the synovial fluid, small crystals are located in the center and large along the periphery. Spectral analysis of crystals revealed high Na content. In pyrophosphate arthropathy, a large number of crystals of various sizes are located randomly. Smears spectral analysis showed the presence of calcium in only four out of 18 patients. The largest crystal size (up to 4 microns) was observed in synovial fluid in arthrosis. Crystals spectral analysis showed a greater percentage of Cl and Na. Rheumatoid arthritis is characterized by conglomerates of small crystals and a high Na content in comparison with other elements. The obtained features of SEM images and spectral analysis of the chemical elements of crystals may be important in the future for differentiating the diagnosis and may become the basis for the development of new diagnostic methods.

KEYWORDS
Arthritis, Synovial Fluid, Microcrystals, Sem Images, Spectral Analysis.

Introduction

In recent decades, incidence of arthritis in the world and in the Russian Federation is increasing. In the Republic of Sakha (Yakutia), rheumatic disease prevalence is 1.5-2 times higher than the national figures [1].

Identification and crystals morphology characterization of synovial fluid is of importance in the diagnosis of joint diseases. Currently, there are various methods for diagnosing synovial fluid. But in practice, developing optimal diagnostic algorithm, to make a reliable diagnosis, is difficult due to the limited possibilities of existing techniques. Abilities of optical microscopy are limited because spatial resolution, increase in the studied objects associated with the physical properties of light (wavelength) do not allow studying the morphology of biological objects at the nanometre level. The urgency of the problem is associated not only with an increased incidence, but also with the difficulty of identifying crystals in various joint pathologies, including early diagnosis.

Microcrystalline arthritis is a group of diseases caused by inflammation in the places of crystal deposition formed in vivo. Although the pro-inflammatory potential for different crystals differs (sodium monourate, calcium pyrophosphates (PFC), hydroxyapatite, etcю crystals), all of them can be clinically manifested by acute inflammation, more often arthritis or periarticular lesion of one or more joints. Most often, arthritis is caused by urate crystals (gout) and calcium pyrophosphate. PFC crystals in synovial fluid is confirmed by polarizing light microscopy in the form of characteristic crystals (in the form of a parallelepiped, mainly intracellular, with little or no light reflection). It is necessary to consider that polarized light analysis reveals approximately 1/5 of all PFC crystals. Recently, to visualize PFC crystals, luminescence microscopy using a calcium-sensitive fluorescent probe Fluo-4 was suggested. This method also allows flow cytometry for rapid semi-quantitative crystal analysis. Experienced researcher, can identify PFC crystals by conventional light microscopy (unlike needle-shaped urates, they look like parallelepipeds with "chopped off" ends, rhombuses). A more reliable method for detecting and identifying crystals is microscopy in polarized light using a compensator, in which the ability of pyrophosphate crystals to weakly double refraction is revealed [6].

Crystal analysis the with a polarizing microscope shows crystals of calcium pyrophosphate dihydrate as rhomboid and positive birefringent (as opposed to negative birefringent needle crystals of urate) [9] (Fig. 1). This test is done in

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most commercial and institutional laboratories, but even in the best cases, results can be false negative (too few crystals) or false positive (artifacts or debris can mimic crystals) Polarized light can detect calcium pyrophosphate crystals 5 times more often than with conventional microscopy.

Another type of crystalline arthritis is gout (Greek ποδάγρα, - foot trap) [7]. This is a metabolic disease characterized by the deposition of urate crystals in various tissues of the body in the form of sodium monourate or uric acid. The occurrence is based on the accumulation of uric acid and its decreased excretion by the kidneys leading to its increased concentration in the blood (hyperuricemia). Gout has been known since ancient times. The first written evidence of the disease comes from Ancient Egypt and dates back to 2600 BC They are based on the description of the thumb gouty arthritis [8]. In the 5th century BC, Ancient Greek healer and physician Hippocrates described the clinical symptoms of gouty arthritis in his "Aphorisms", where he noted that the disease does not occur in eunuchs and women before menopause [9,10]. In 1679, the Dutch scientist Anthony van Levenguk for the first time described the microscopic structure of uric acid crystals [11]. In 1848, the English physiologist Alfred Baring Garrod, using a thread dipped into the blood of a patient suffering from gout, discovered and described an increased blood uric acid level in this disease [12-14]. In 1899, urate crystals were found in the articular fluid during gouty arthritis attack. In 1961, MacCarty and Hollander determined the role of urate crystals in the onset and development of gouty inflammation [15]. Urate crystals are in the form of rods or thin needles with broken or rounded ends about 10 microns long. Urates microcrystals in synovial fluid are found in acute, less often subacute arthritis, both free-lying and in neutrophils.

Plasma is considered to be saturated with urates when its level reaches the solubility limit of about 6.8 mg / dL (405 μmol / L) [10-1]. If this concentration is exceeded, the solution is considered to be oversaturated. In the supersaturation range, MSU crystal formation begins with a further change in urate solubility or other triggering event, and further crystallization spreads depending on local conditions.

In the literature available, there are no studies of synovial fluid microcrystals by electron microscopy. Joints puncture with the subsequent examination of the synovial fluid is an irreplaceable diagnostic method that, in most cases, allows to identify various crystals and to recognize gout and PFA even in the interictal period using the correct methodological approach.

The aim of the study was to characterize synovial fluid crystals in various pathologies of the knee joint using a scanning electron microscope with energy dispersive analysis of spectrum elements using the INCA Energy system.

Material and Methods

The studies were performed on a high-resolution scanning electron microscope (SEM) with a thermal-field Schottky cathode JSM-7800F (Japanese Electron Optics Laboratory - JEOL, Japan), which is used for research and analysis of nanostructures, determination of elemental composition, with a resolution of 1.2 nm (at 1 kV) with the GentleBeem system in the mode of low accelerating voltages (1-2 kV) without conductive coatings of the samples. This study examines SEM images obtained by detecting secondary electrons from samples using the lower detector of the microscope. Such accelerating voltages and operation in the mode of deceleration of the beam "GentleBeam" make it possible not to cover the surface of the samples with a conductive layer, which increases the possibilities of improving the image quality at such low accelerating voltages and with the simultaneous use of the deceleration system of the beam electrons immediately before they fall on the sample under study [21].

This SEM is retrofitted with the Oxford INCA Energy 350 energy dispersive microanalysis system. The sample diameter should not exceed 125 mm. The analysis area ranges from 1mcm^2 to 20mcm^2. Additional information was obtained using the data of the energy dispersive microanalysis Oxford INCA Energy-350, which allows to estimate the ratio of macroelements and their spectrum.

The object of the study was dry smears of synovial fluid obtained by puncture of the knee joint of 101 patients with various diagnoses: rheumatoid arthritis - 25, gout - 18, reactive synovitis - 15, osteoarthritis - 25, pyrophospatous arthropathy - 18.

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Results and Discussion

Analysis of SEM images of synovial fluid in various forms of arthritis is characterized by various shapes and sizes of crystals, spatial arrangement of conglomerates, depending on the diagnosis, flow form. Attempt to assess the ratio and spectrum of macroelements in the induced crystal was made.

Uric acid, the end product of purine metabolism, is known to be a weak acid that circulates in the form of deprotonated urate anion under physiological conditions and combines with sodium ions to form sodium monourate. Sodium monourate crystals are known to have a triclinic structure, in which sheets of purine rings stacked on top of each other form needle-like crystals, observed under a microscope (9-1). The exposed charged crystal surfaces are thought to mediate interactions with phospholipid membranes and serum factors that play a role in the crystal-mediated inflammatory response (10-1). Although hyperuricemia is a gout certain risk factor, local factors such as temperature, pH, mechanical stress, cartilage components, and other synovial and serum factors have been suggested to play a role in crystal formation. Interestingly, crystals of sodium monourate can stimulate the generation of crystal-specific antibodies that facilitate future crystallization of sodium monourate (8-1).

SEM images of the synovial fluid of a patient with gout are characterized by a large number of crystals compared to smears from patients with other forms of arthritis (Fig. 1). It should be noted that no crystals of monourates in the form of sticks or thin needles were found.

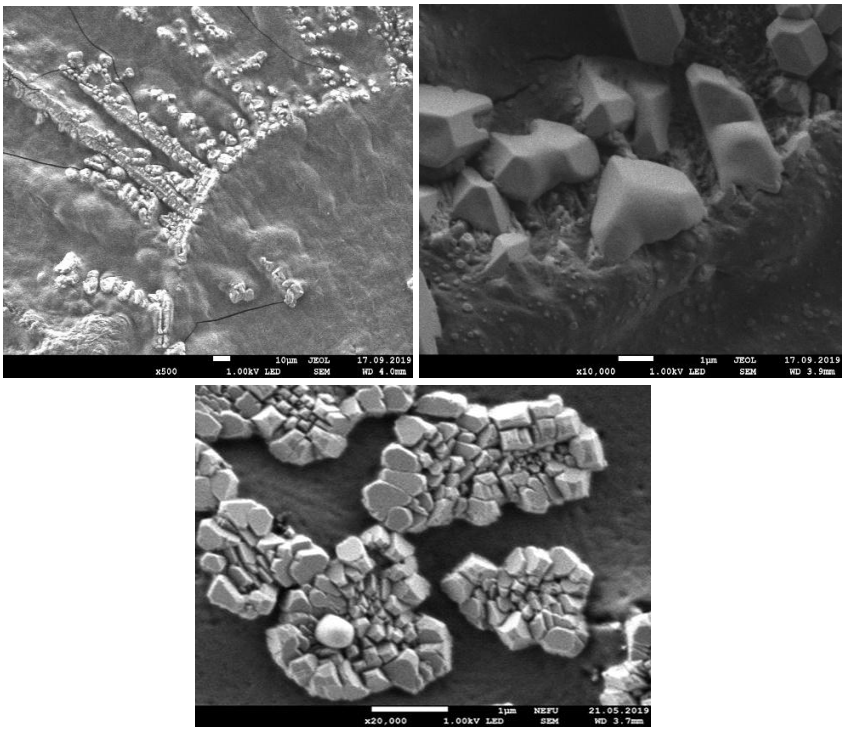

Fig. 1. SEM images of a dry smear of synovial fluid at various magnifications: a) x 500; b) x 10000; c) x 20,000.

Mainly crystals of two types were seen in the images: - small, the size of which ranged from 0.1 to 0.3 mcm; - large with sizes from 0.6 to 2.0 mcm. It should be noted that SEM images for gout differ from others in the particular ordering of the crystals relative to each other. A characteristic pattern is observed: in the center there are smaller crystals, and along the periphery - large ones (in the form of a flower with petals).

According to the literature, during the "nucleation", dispersed molecules in solution are first collected in clusters, overcoming the dispersion forces of the solvent. Then these clusters combine to form crystal cores [13-1]. When the urate crystal core reaches a critical size and its primitive structure stabilizes, crystal growth occurs most rapidly at the

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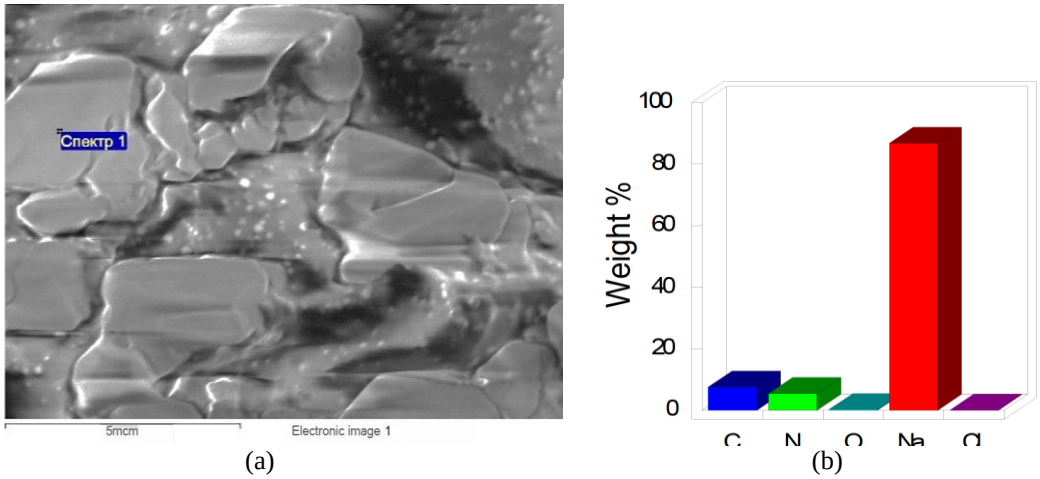
longitudinal ends of the crystal. It is this predisposition to longitudinal over transverse growth that gives the sodium monourate crystal its characteristic long, narrow shape. Although the mechanisms of crystal growth remain unresolved in vivo, the growth of urates under model conditions was compared with recent data suggesting that the spontaneous appearance of islands on crystal surface may play a role in crystal growth [19].

Under normal physiological conditions (at pH 7.4 and 37 ° C), urates are known to circulate in plasma and synovial fluid in a monodeprotonated ionic form. In the crystalline state, urates are observed either in the form of a fully protonated acid (for example, uric acid, as in the case of kidney stones), or in the form of different salts formed by deprotonated or partially deprotonated urate. Sodium urate monohydrate (NaC5 H3 N4 O3 · H2O), in which urate molecule is bound to one sodium molecule and one water molecule, is one of the most common forms of crystallized urates and includes the primary deposits observed in gouty arthritis [11-1.], although the precipitation of urates with other mineral phases is possible [12-1].

Spectral analysis of the elements of one of the induced large crystals (Fig. 2a) shows the following ratio of elements: Na, C, N, O, and Cl. In this crystal, the predominant element is Na (weight - 86.8%, atomic - 78.6%), then C (weight - 7.54%, atomic 13.06%), N (weight - 5.41%, atomic - 8.04), O (weight - 0.23%, atomic - 0.31%) Fig. 2 b).



(a) (b)

Fig. 2. a) SEM image of the selected crystal with the text “Spetrum1”; b) the quantitative results of the smear element analysis in gout

In our study the spectral analysis if the induced large crystal is indirectly comparable with the element composition of sodium monourate. Thus, the spectral analysis of the “polarized” arrangement of the crystals relative

Another type of microcrystalline arthritis is the so-called pyrophosphate arthropathy (pseudogout), which is accompanied by the formation of specific calcium pyrophosphate crystals, presumably resulting from changes in pyrophosphate metabolism in the articular cartilage. Recently, the role of calcium pyrophosphate (PFC) dihydrate crystals in the development of musculoskeletal locomotor system pathology is thoroughly studied. The prevalence of diseases associated with the deposition of calcium crystals increases with the age of patients, amounting to 10-15% in patients 65-75 years old and reaching 40% in people over 80 years old. The concept of the frequency occurrence of pyrophosphate arthropathy is rather arbitrary, since the disease can be masked by osteoarthritis (OA), rheumatoid arthritis, gout, manifest as undifferentiated arthritis, which complicates its timely diagnosis [2].

PFC crystals in cartilage and synovial fluid in OA were determined in 65% of patients with gonarthrosis [3,4]. PFC crystals are primarily deposited in the thickness of the articular cartilage, then on its surface and then in the synovial and periarticular tissues [5].

Fig.3 shows SEM-pictures of synovial fluid sample with pyrophosphate arthropathy at a magnification of 10,000 and a selected crystal for element analysis with text “Spectrum 1” pointed at it.

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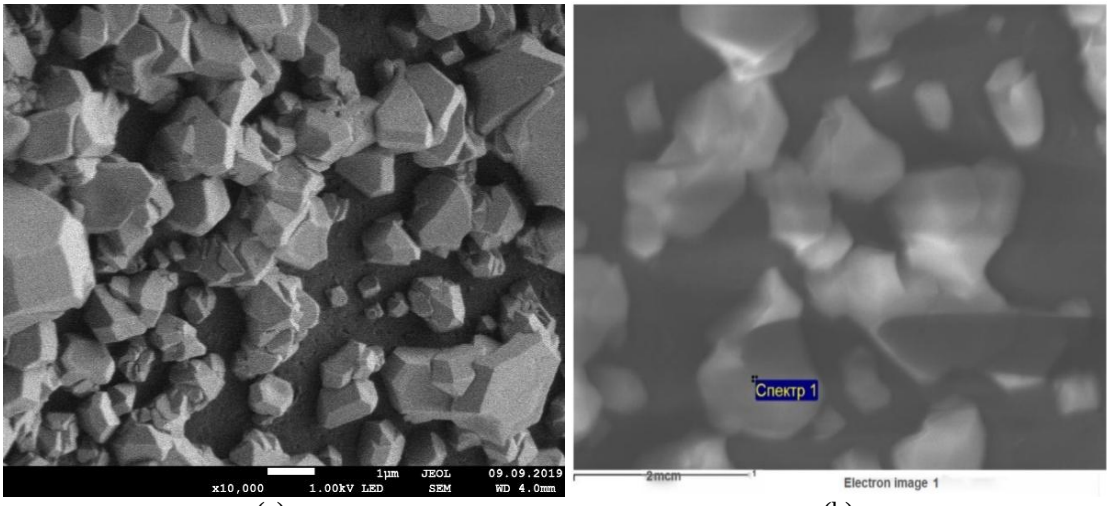
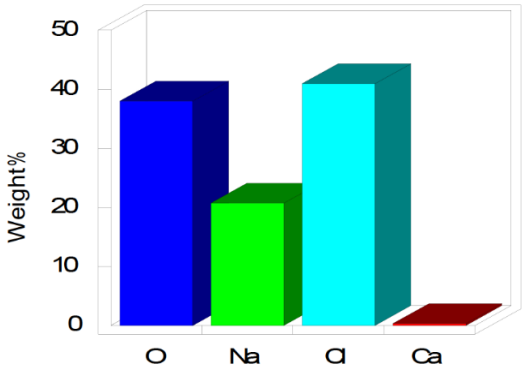


(a) (b)

Figure 3. a) SEM image of the synovial fluid crystals of a patient with pyrophosphate arthropathy at a magnification of 10,000; b) SEM image of the selected largest crystal with the text "Spectrum 1".

In this arthropathy, many crystals with large, medium and small sizes, arranged irregularly, are characteristic. Spectral analysis of synovial fluid smears of 18 patients with a preliminary diagnosis of pyrophosphate arthropathy showed the presence of calcium in only four patients. In the rest 13 smears, the elemental composition was similar to the results of spectral analysis of patients diagnosed with osteoarthritis of unknown etiology. Spectral analysis indicates the predominance of the elements Cl, O and Na in the crystal. Calcium pyrophosphate (diphosphate) dihydrate, $Ca_2(P_2O_7)_2H_2O$, is a natural inorganic calcium salt present in body tissues (Fig 4).



Element%	Weight%	Atomic%
O K	38.02	53.51
Na K	20.71	20.29
Cl K	40.95	26.02
Ca K	0.32	0.18

Fig. 4. Quantitative results of the element analysis of a crystal sample of the synovial fluid from patient with the diagnosed pyrophosphate arthropathy

Fig. 5 and 6 show the results of a study of a synovial fluid sample in a patient diagnosed with arthrosis of the knee joint. The SEM image shows conglomerates with a predominance of larger crystals (up to 4 μm). The results of the spectral elemental analysis of the selected large crystal with the induced text "Spectrum1" indicates the accumulation of sodium chloride salt in the synovial fluid, quantitative analysis indicates the predominance of chlorine over sodium.

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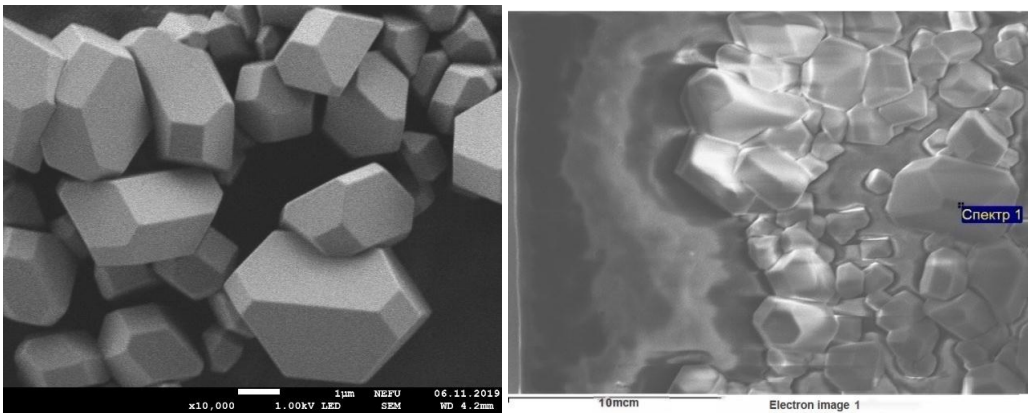
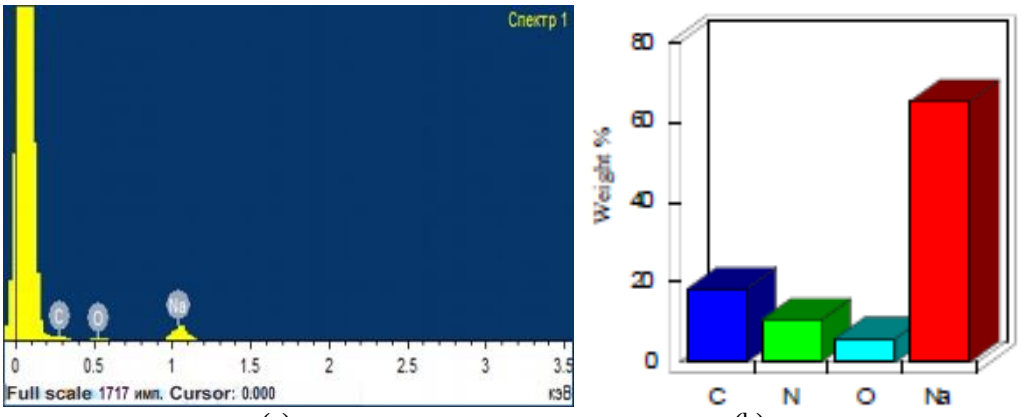


(a) (b)

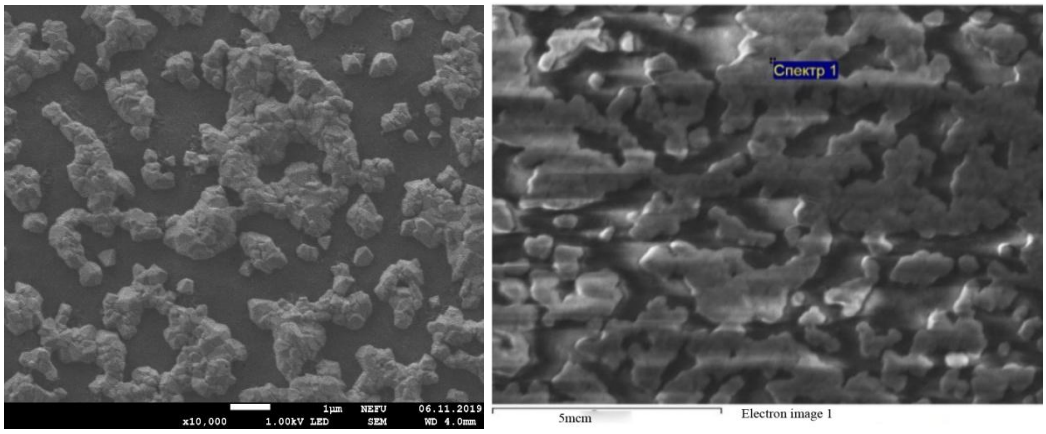
Figure 5. a) SEM image of synovial arthrosis crystals at a magnification of 10,000; b) SEM image of the selected largest crystal with the text "Spectrum 1".



(a) (b)

Fig. 6. a) spectrum of crystal elements in arthrosis b) quantitative analysis of crystal elements in percent

Fig. 7 and 8 show SEM images and the results of spectral analysis of a patient diagnosed with rheumatoid arthritis. It should be noted that in this pathology, the synovial fluid is represented by a dense network of conglomerates (sodium chlorine), consisting of many small crystals (0.3 - 0.5 microns) and most likely in a complex with organic substances, which is a matrix that adheres the crystals.



(a) (b)

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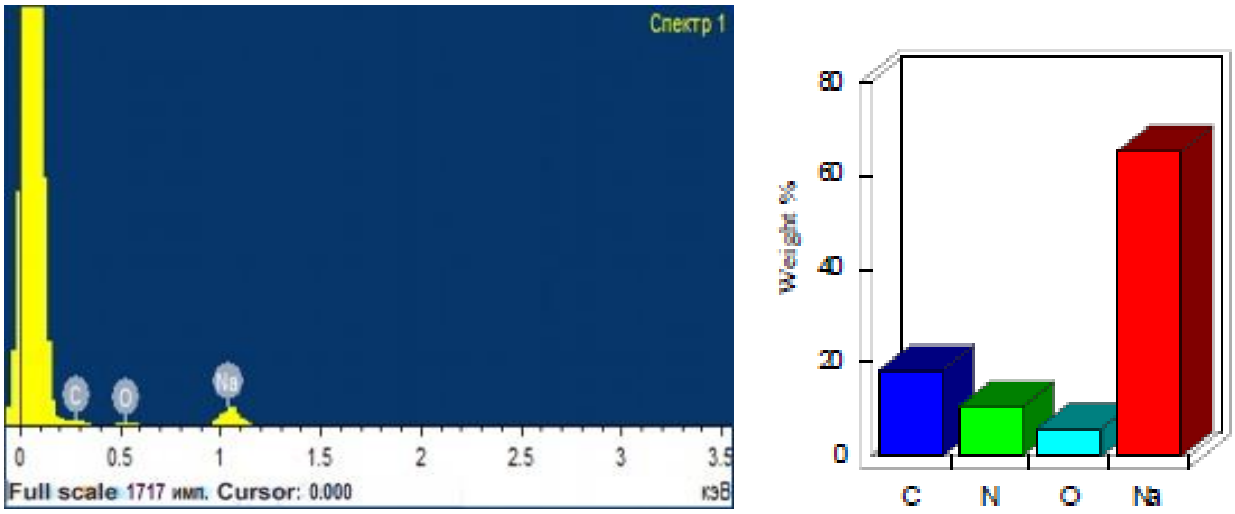
Fig. 7. a) SEM image of synovial fluid crystals in rheumatoid arthritis at magnification of 10,000; b) SEM image of the selected crystal with the text "Spectrum 1"

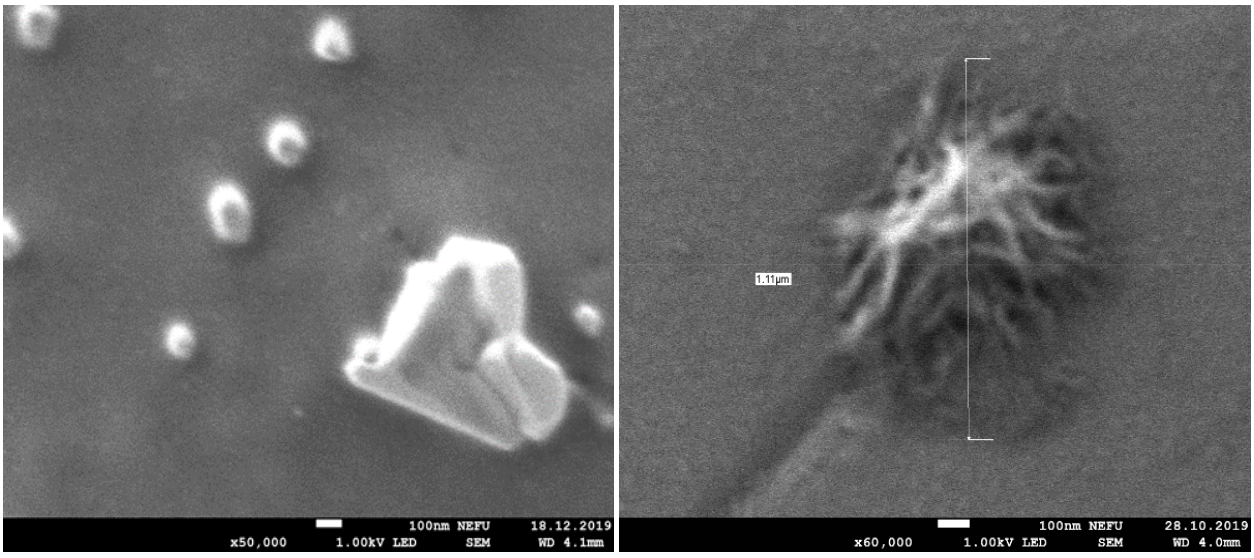
Spectral analysis of the induced crystal is characterized by the predominant Na content (wt% - 65.22; atomic% - 51.2%).



(a) (b)

Fig. 8. a) spectrum of crystal elements in rheumatoid arthritis; b) quantitative analysis of crystal elements in percent.

In some samples of synovial fluid with various pathologies of the knee joint, SEM images, in addition to crystals, revealed objects that correspond in size to viruses and bacteria. The joint infection is known to occur not only in reactive arthritis. Fig. 9 shows an object similar in size (about 1 μm) and shape to a bacterium and nanoparticles, presumably viruses in size.



(a) (b)

Fig. 9. SEM images of: a) the alleged bacterium at a magnification of x60,000, indicating its size; b) allerged viruses at a magnification of 50,000

Fig. 10 presents a diagram of quantitative elemental analysis of a nanoparticle - alleged virus. Quantitative spectral analysis shows the organic nature of this nanoparticle and indirectly confirms our assumption on their viral nature.

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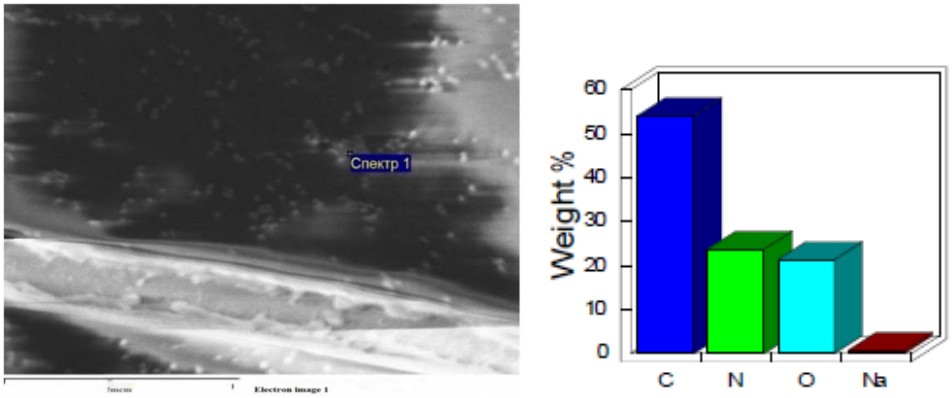

(a) (b)

Fig. 10. SEM images of a synovial fluid sample: b) Weight, atomic percentage distributions of nanoparticle elements in a synovial fluid sample

Conclusion

The obtained SEM images allowed to study in more detail the structure, shape, size and features of microcrystals location in samples of synovial fluid from patients with various diagnoses of joint pathology. The obtained features of SEM images and spectral analysis of chemical elements of crystals may be important in future for differentiating the diagnosis and may become the basis for the development of new diagnostic methods. The quality of SEM images at much higher magnifications compared to optical microscopes and the simultaneous possibility of elemental analysis increase the reliability o rheumatic disease diagnosis. SEM was shown to be a very informative technique for examining patients with rheumatic diseases. The prospect of its is in the identification of these diseases at early stages and the possibility of a more detailed assessment of the treatment dynamics.

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