METHODS

Simple Method of Specimen Preparation for Scanning Electron Microscopy

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 151, No. 3, pp. 356-360, March, 2011
Original article submitted March 24, 2010

We compared conventional method for specimen (cell cultures, tissue specimens) preparation for scanning electron microscopy and a method without sputtering and critical-point drying. OTO-method (osmium-thiocarbohydrazide-osmium) with sample impregnation with hexamethyldisilazane followed by air drying was used as an alternative method. Excellent preservation of surface ultrastructures and electrical conductivity was proved. The method is easy to use and does not require additional costs for equipment.

Key Words: *scanning electron microscopy; OTO-method (osmium-thiocarbohydrazide-osmium); specimen preparation; cell culture*

Conventional sample preparation for scanning electron microscopy (SEM) includes fixation, dehydration, drying, and sputtering. First two stages are the same for both transmission electron microscopy and SEM and can be combined in case of complex investigations.

Specimen drying for SEM is a critical stage, because surface tension occurring during evaporation of dehydrating fluids, such as alcohol and acetone, during air-drying results in substantial and irreversible deformation of structures. This can be avoided by using critical-point drying. In this case, acetone (or alcohol) is substituted with liquid carbonic acid, the specimen is transferred to the critical point where carbonic acid immediately transits into gaseous state. It eliminates destructive effects of surface tension and structure surface remains in native condition. Devices for drying at the critical point are relatively expensive (up to $20,000), and active search of the compounds that allow abandonment of this drying method was carried out for the last 10 years. Currently, the compound hexamethyldisilazane is found, which is organosilicon monomer freely mixable with alcohol and acetone and polymerizing with no effect on surface tension [2]. It enables to abandon the conventional drying at the critical point and to reduce costs (time and finances) on specimen preparation for scanning electron microscopy.

The second critical stage in specimen preparation is to make the specimen electroconductive. For this purpose, metal sputtering under the vacuum is traditionally used (gold ion sputtering or carbon-platinum sputtering). These effective methods are also expensive. Their main disadvantage consists in the fact that only the surface of the specimen becomes electroconductive. We propose OTO-method (osmium-thiocarbohydrazide-osmium) as an alternative [4]. This method includes double osmium coating with thiocarbohydrazide impregnation. An advantage of this method is bulk specimen electroconductivity, what allows employment of micromanipulators integrated into scanning electron microscope for specimen breakage

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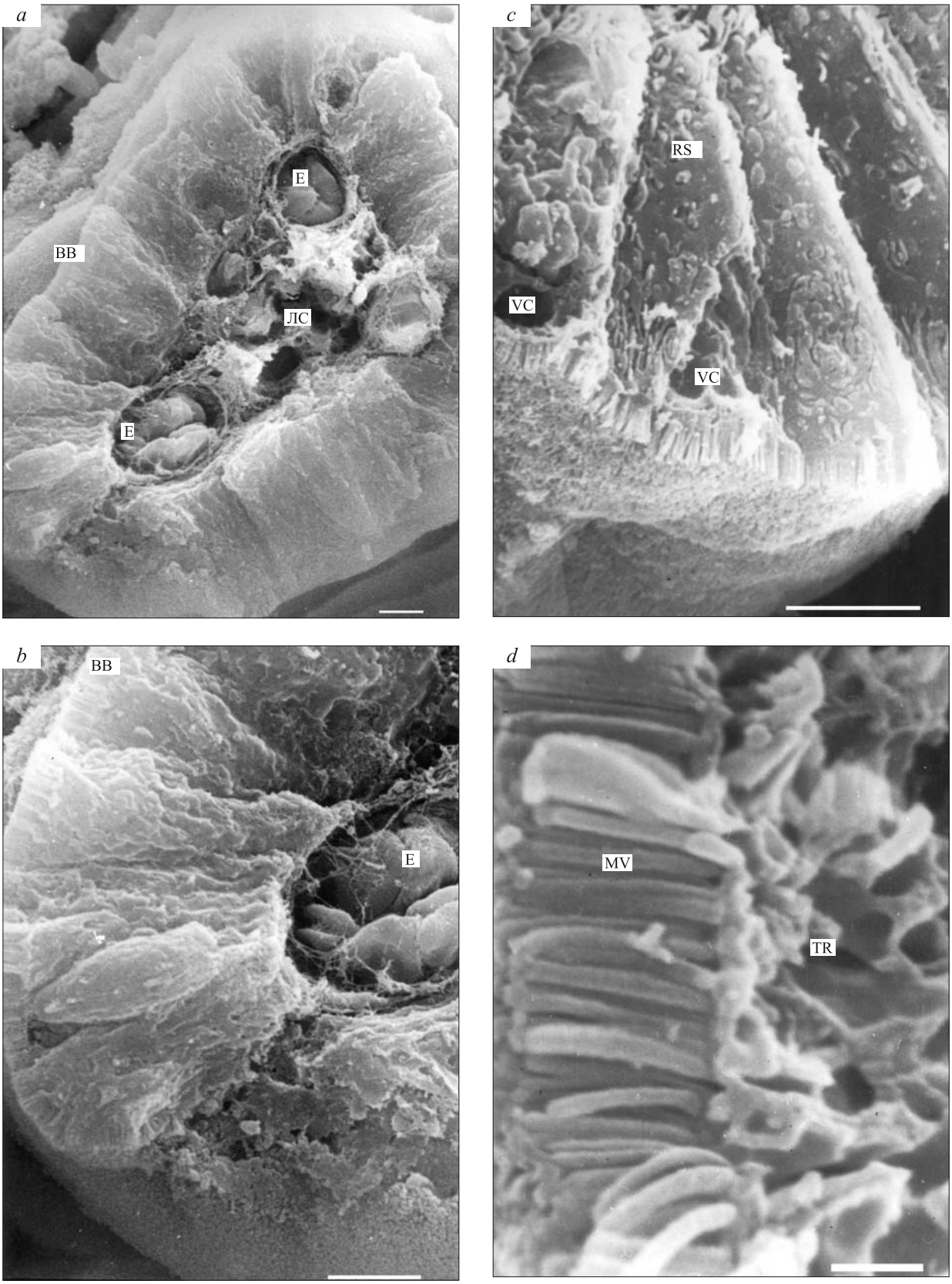

Fig. 1. Break of the villus of the sucker rabbit small intestine prepared using OTO-method without gold sputtering. BB: brush border, RS: rough surface of the epithelial cell, MV: microvilli, VC: vacuoles, TR: terminal reticulum, E: erythrocytes. Here and in Fig. 3: scale: 5 and 0.5 μ, respectively.

under the control of electron beam and investigation of 3D-structure of cell organelles.

The objective of our study was to develop the device-free method of specimen preparation for SEM.

MATERIALS AND METHODS

Small intestine from sucker rabbits, uterine endometrium biopsy specimens, and culture of mesenchymal stem cells (MSC) were used in the study. The specimens were prepared routinely: tissues and cell culture were fixed in 2.5% glutardialdehyde on 0.1 M phosphate buffer for 1 h at room temperature, and then postfixed in OsO_4 (1% aqueous solution on 0.1 M phosphate buffer, 1 h at room temperature), and dehydrated in ascending acetone concentrations. Dehydrated specimens were dried at a critical point in liquid CO_2 in Polaron device and sputtered with gold (100 nm thick) using ion sputtering technique in Eiko device. In parallel, the same material was prepared using OTO-method. For this purpose, aldehyde fixation and postfixation in OsO_4 were followed by culture impregnation with 1% thiocarbohydrazide aqueous solution (1 h) at 50°C and one more osmium treatment. After dehydration, the specimens were impregnated with hexamethyldisilazane monomer (Sigma) and air dried.

RESULTS

The proposed methods for specimen preparation for SEM showed good results similar to those obtained by conventional methods (critical-point drying in liquid CO_2 and gold sputtering).

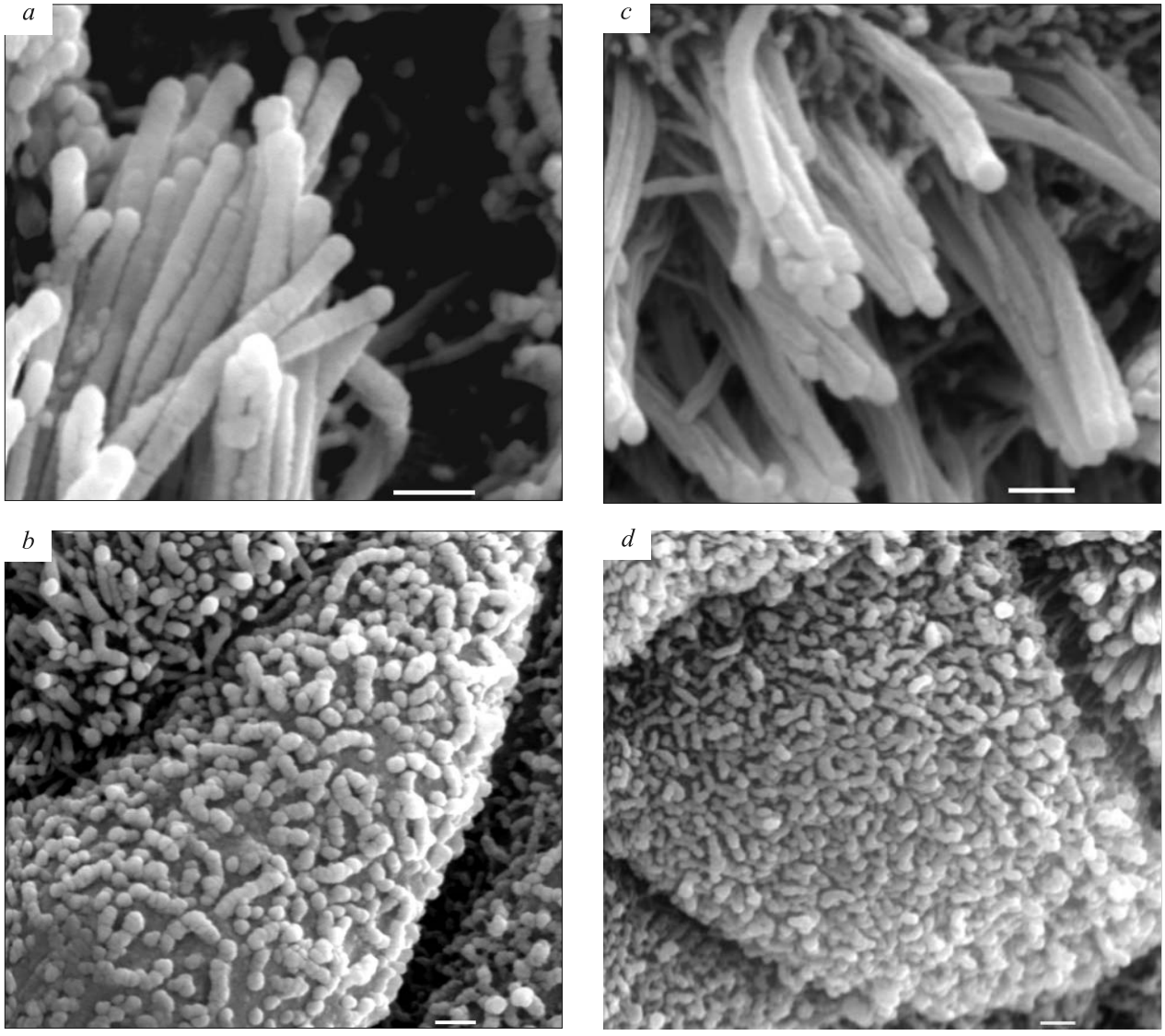

Fig. 2. Uterine epithelium prepared for SEM using hexamethyldisilazane without critical-point drying (*a*, *b*). Uterine epithelium prepared using conventional method with critical-point drying (*c*, *d*). Scale: 0.5 μ.

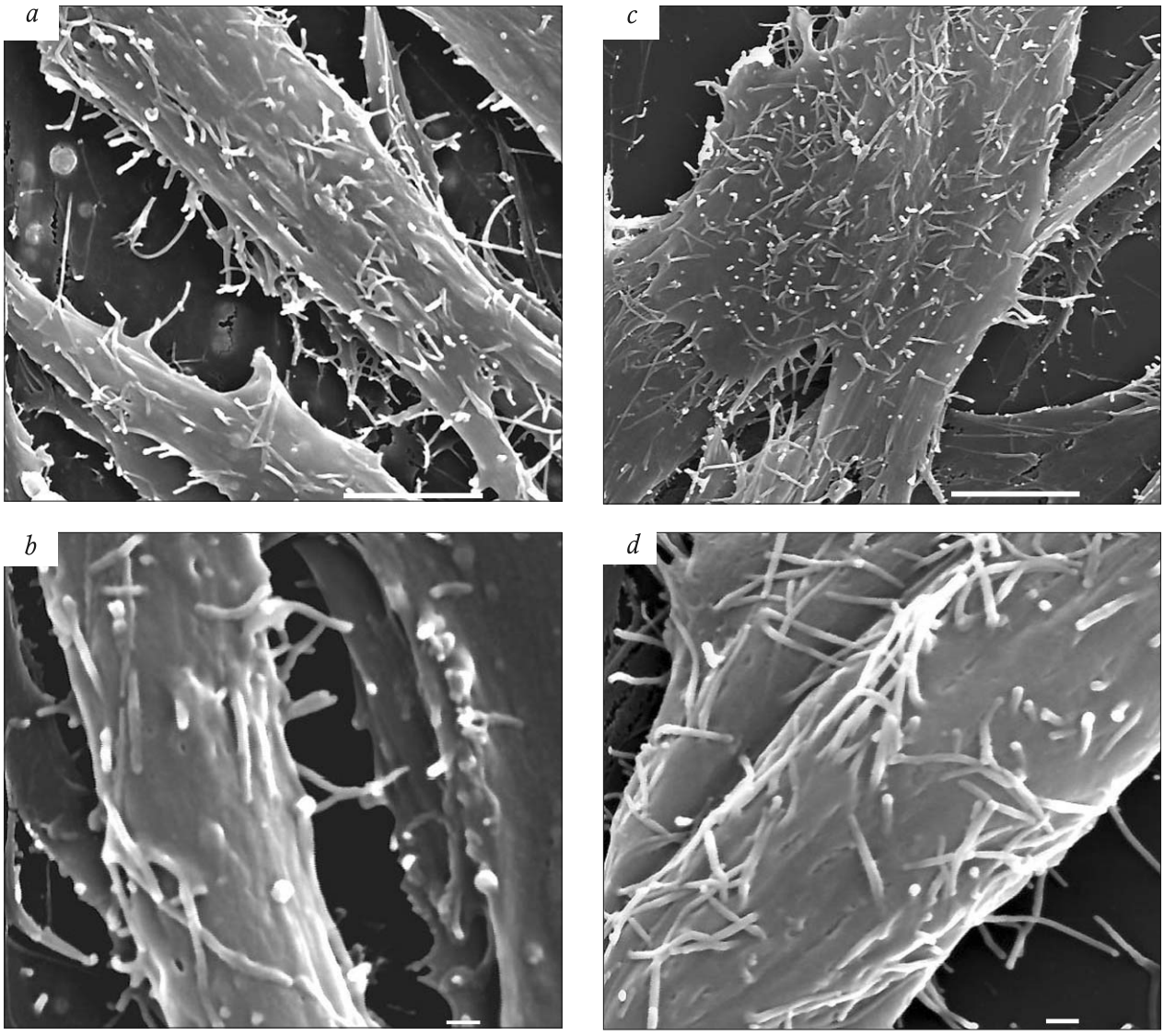

Fig. 3. Mesenchymal stem cell culture prepared for SEM using conventional method (*a*, *b*) and without critical-point drying (*c*, *d*).

SEM of the small intestine surface of rabbit-sucker after OTO treatment are present (Fig. 1). The villi were broken using special micromanipulator under the control of an electron scanning microscope. Enterocyte surface with microvilli as well as the inner surface and intracellular structures (vacuoles, terminal reticulum) were seen. At the same time, it was difficult to predict how villus breakage will appear and what intracellular structures will become available for the analysis, since it is defined by mechanical properties of the cell and appear to be a stochastic process.

Comparison of conventional method for preparation (critical-point drying in liquid CO_2 with ion gold sputtering) and method used in this study (OTO-method with air drying after impregnation in hexamethyldisilazane) revealed no differences in preparation structure in both uterine epithelium preparation (biopsy material) and in MSC culture (Fig. 2, 3). The specimens became electroconductive and did not require sputtering, while air drying after impregnation with hexamethyldisilazane did not result in destruction under the effect of surface tension.

OTO-method was widely used in transmission electron microscopy to increase the contrast during identification and detection of actin filaments in cultures of rat fibroblasts and human monocytes [1]; it was also noted that simple osmium treatment results in their destruction. In addition, the use of OTO-method significantly increased contrasting of other intracellular structures (mitochondria, nuclei) [7].

Previous comparative study of routine drying method in CO_2 and air drying after hexamethyldisilazane impregnation of rat rectal carcinoma revealed no differences [5].

The method of double osmium treatment was also associated with better preservation of unsaturated

lipids during investigation of rat peripheral nerves in scanning microscope [3]. The efficiency of the OTO-method was also shown in cell culture examination under both scanning [6] and transmission electron microscopes [8].

The proposed alternative approaches significantly simplify specimen preparation for SEM and reduce the costs of experimental studies.

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