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Agarose Cell Block: Innovated Technique for the Processing of Urine Cytology for Electron Microscopy Examination

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Easy manipulation and preservation of cells in suspension through the different steps of sample processing for electron microscopy examination is essential for proper diagnosis. The author used agarose gel as an embedding media for processing cells in suspension for electron microscopic examination. The AgarCyto cell block procedure of Kerstens et al. (J Histochem Cytochem. 2000; 48: 709—718) was used to begin electron microscopic processing of exfoliated urothelial cells in voided urine or cells in suspension. Processing of agarose cell block simultaneously for light and electron microscopic examination represents a great advantage offered by this innovated technique.

Keywords agarose cell block, electron microscopy, urine cytology

Cytological smears are used as a screening process to assist in the early diagnosis of diseases prior to the development of symptoms and thereby enable effective treatment [1]. The use of electron microscopy in the study of cell morphology also became an open prospect for studying early cellular changes (atypia, neoplasms) [2]. Sedimentation of maximum number of exfoliated urothelial cells in voided urine with optimum cellular preservation is critical for proper cytological diagnosis, especially at an ultrastructural level.

Cell in suspension (such as fine-needle biopsy aspirates, bone marrow specimens, or cytology samples) are best embedded in a protein support medium before processing for electron microscopic (EM) examination. Blood plasma, agar, or bovine serum albumin (BSA) were used for this purpose [3]. Cell blocks can also be prepared from fluid specimens.The most widely used method employs 2% agar for light cytopathological examination [4].

The present work examines the possible use of agarose gel as an embedding media for processing urine samples for EM examination. This idea is based on the Agarcyto technique of Kerstens et al. [5], which was based on the protocols of Olson et al. [6] and Kung et al. [7], who used agar as a cellular embedding media. The hypothesis of this study depended on the easy manipulation of agarose at a low melting point. This can offer a good chance for cellular sedimentation in the liquid form of the embedding media before its consolidation.

MATERIAL AND METHODS

The material of this study consisted of voided urine samples. They were harvested from 40 cases in the outpatient clinic and urology department of Theodor Bilharz Research Institute Hospital. Twenty of these cases had bladder carcinoma, diagnosis was histopathologically confirmed by using cystoscopic bladder biopsy. The other 20 cases were diagnosed clinically as acute cystitis, with accompanying suprapubic pain, dysuria, frequency, and urgency.

The amount of the collected voided urine samples ranged from 20 to 30 mL. Twenty cases (10 malignant and 10 acute cystitis) were processed for electron microscopic examination by using 2 conventional techniques: the direct technique and the agar technique. In the direct technique, processing of urine samples was done without using any supporting media. The other 20 cases were processed using the innovated agarose cell block technique. (Many trials have preceded this comparative study to adjust the optimum conditions of the agarose cell block (ACB) technique).

The following common steps were performed for both conventional and innovated techniques. Two conical tubes with 10 mL urine were centrifuged for each case at 1500 rpm for 10 min. The supernatant was poured out. The sediment in the first tube was smeared on a clean slide and processed for Papanicolaou stain (Pap). The sediment in the other tube was manipulated as follows: the sediment with about 1 mL of urine was transferred into an Eppendorf tube and fixed in a

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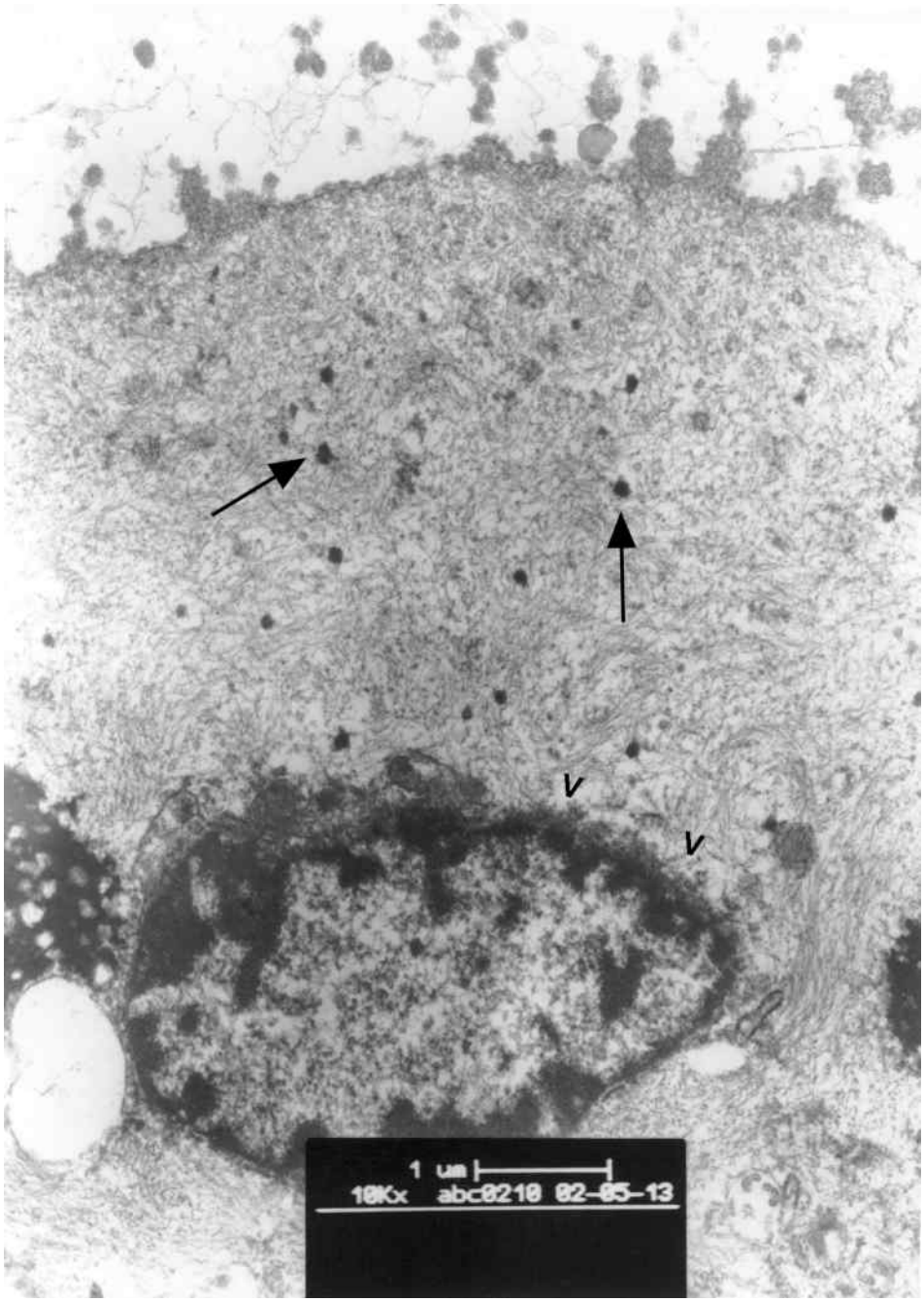

FIG. 1 *Electronmicrograph of urine sample processed by using the ACB technique. Cell membrane and cellular constituents of the malignant cell are clearly evident and well preserved. The cell shows cytoplasmic tonofilaments and increased keratohyaline granules (arrow). The nucleus shows marginating chromatin* (head of arrow). *The cell wall shows short microvilli* (thin arrow). *×10,000.*

mixuture of equal volume of 4% buffered glutaraldehyde and cacodylate 0.2 M for 1 h.

The cell clusters of the samples processed for *direct conventional EM technique* were washed in equal volumes of 0.3 M cacodylate and sucrose 0.4 M, postfixed in 2% osmium tetroxide, dehydrated in ascending alcohol concentration, embedded in epoxy resin, then polymerized. The centrifugation of the sample between each step at 1500 rpm for 3 min was a must to get a sediment before decanting the specific solution.

The cell cluster of the samples processed for EM using the *agar technique* were put in a small hole of about 3 mm in a layer of hardened 1.5% gelose. Another liquefied gelose layer was poured over the site of cell cluster. After solidification of the gelose layer, we cut

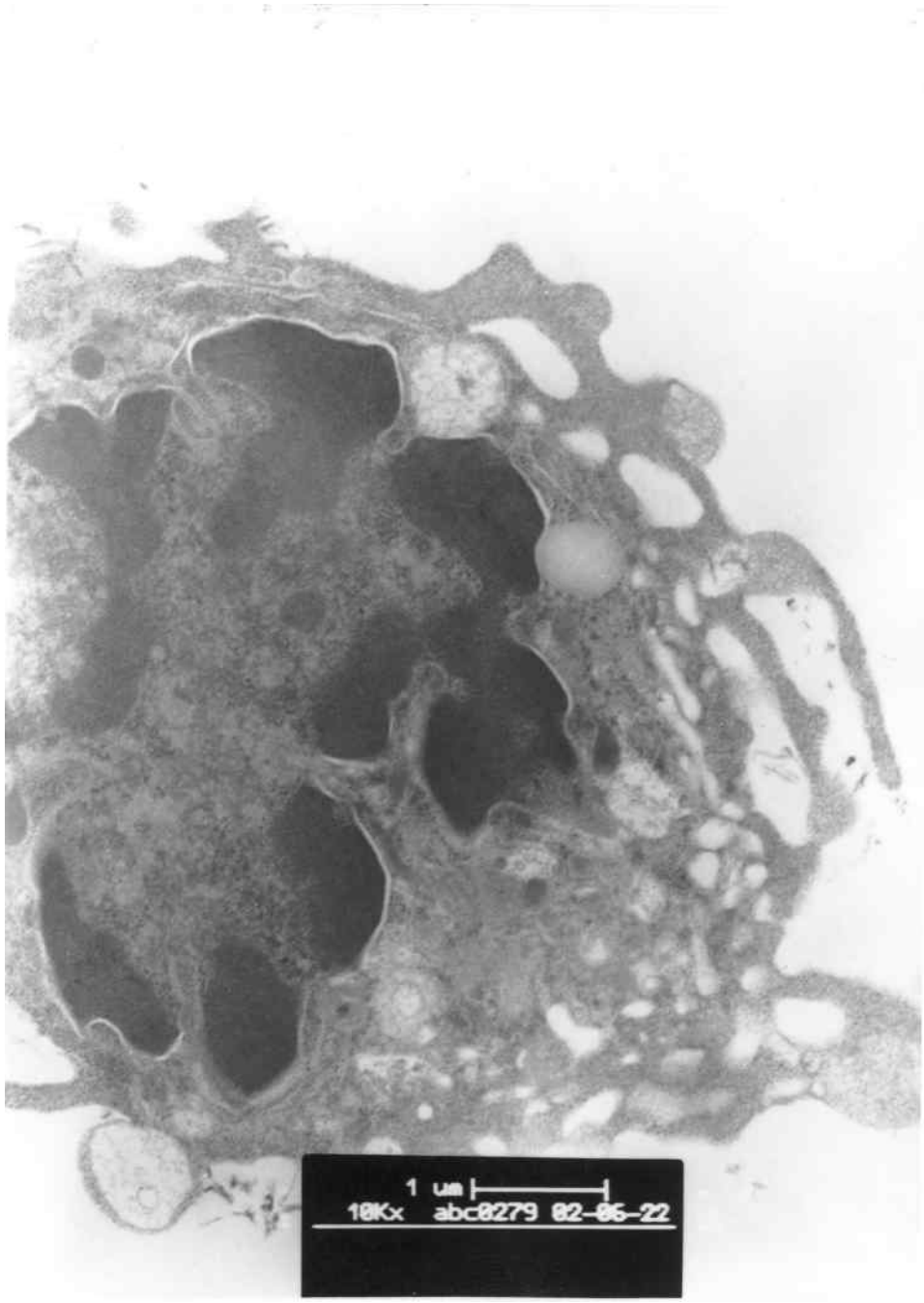

FIG. 2 *Electronmicrograph of urine sample processed using direct conventional EM technique. The cell membrane of the malignant cell is not clearly demarcated as well as the different cellular constituents. ×10,000.*

the gelose containing the burred cells into small cubes, which were manipulated as blocks. The blocks were then processed as the previously mentioned steps. They were postfixed in 2% osmium tetroxide, then dehydrated in ascending alcohol 30, 50, 70, and 95% for 5 min each bath. This was followed by 2 baths of absolute alcohol for 10 min each. The blocks were then embedded in epoxy resin and polymerized at 60°C for 24 h.

The sediment of the samples processed by using the *innovated technique* was resuspended in 1 mL of 2% dissolved agarose in distilled water at 60°C (Agarose [molecular biology grade], Promega, USA). Melting was done using the microwave. The suspension was recentrifuged for 7 min at 1,500 rpm. The agarose with the cell sediment in the cone of the Eppendorf tube was refrigerated at 4°C for 30 min.

The solidified agarose cell block was taken off and the conical part was divided longitudinally into halves. Half was refixed in formalin and processed for the preparation of parrafin blocks, then sectioned into

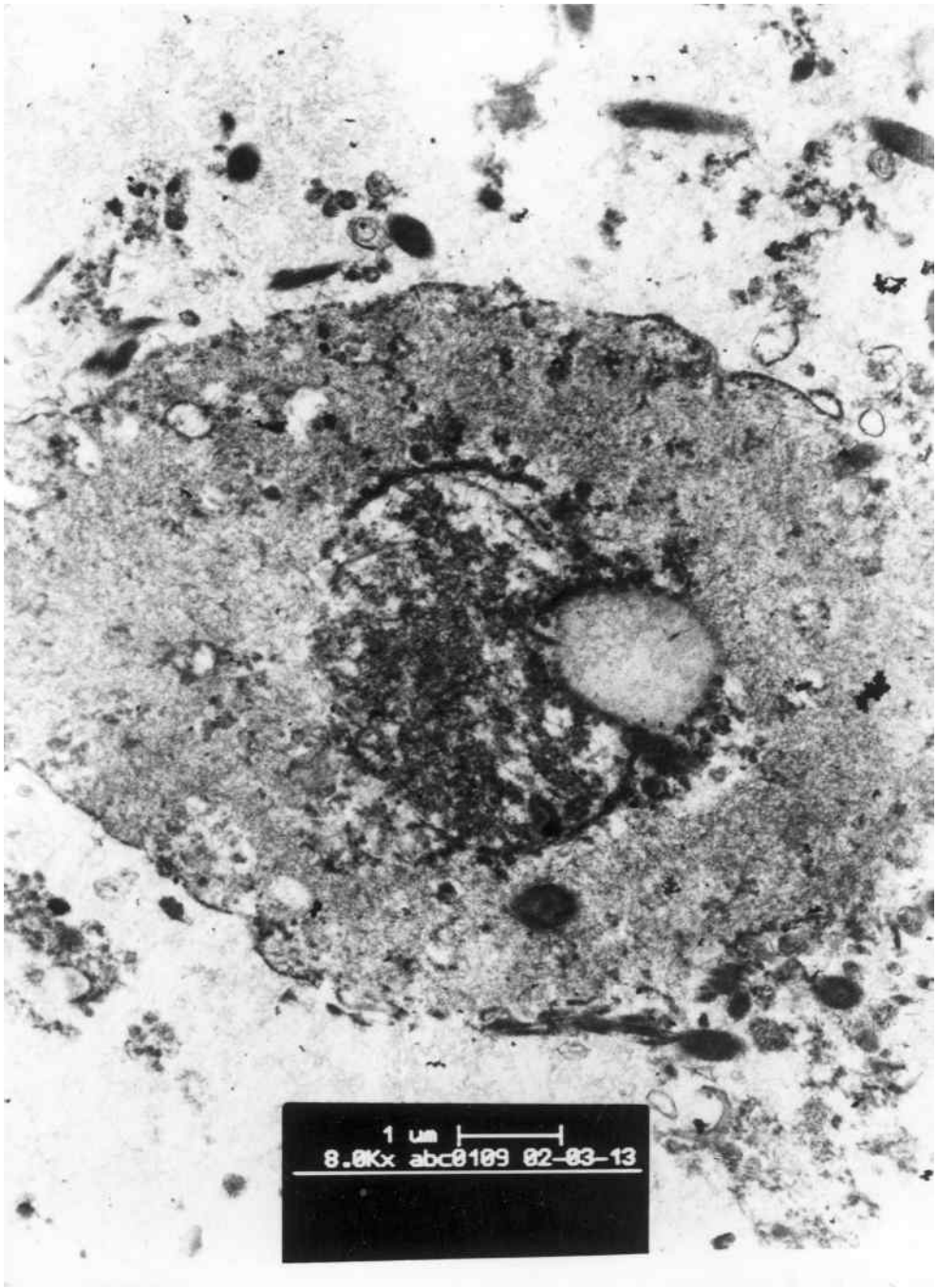

FIG. 3 *Electronmicrograph of degenerated malignant squamous cell processed by using the ACB technique. Note the well-visualized cell remnants and the demarcated parts of cell membrane. ×8,800.*

4-μm-thick sections and stained with hematoxylin and eosin stain. The other half was sectioned into tiny pieces of about 1 mm^3 and transferred to the usual processing container for EM technique. The tiny pieces were refixed in 4% buffered glutaraldehyde in 0.2 M sodium cacodylate for 2 h at 4°C. Fixed sample in buffered glutaraldehyde was washed twice for 2 h each time in an equal volume of sodium cacodylate 0.2 M and sucrose 0.4 M at 4°C. Then it was postfixed in 2% osmium tetroxide for 1 h at 4°C and washed in distilled water. The sample was dehydrated in ascending grade of alcohol 30, 50, 70, 95, for 5 min each bath and absolute alcohol for 3 baths of 5 min each. Then it was infiltrated with equal volume of a mixture of Epon [A and B] and absolute alcohol for 2 h at room temperature, then in a mixture of 75% Epon and 25% absolute alcohol for 2 h, then in equal volume of Epon A and B for 2 baths—one bath over night and the other for 4 h under vacuum to remove bubbles. This was followed by embedding of the sample in

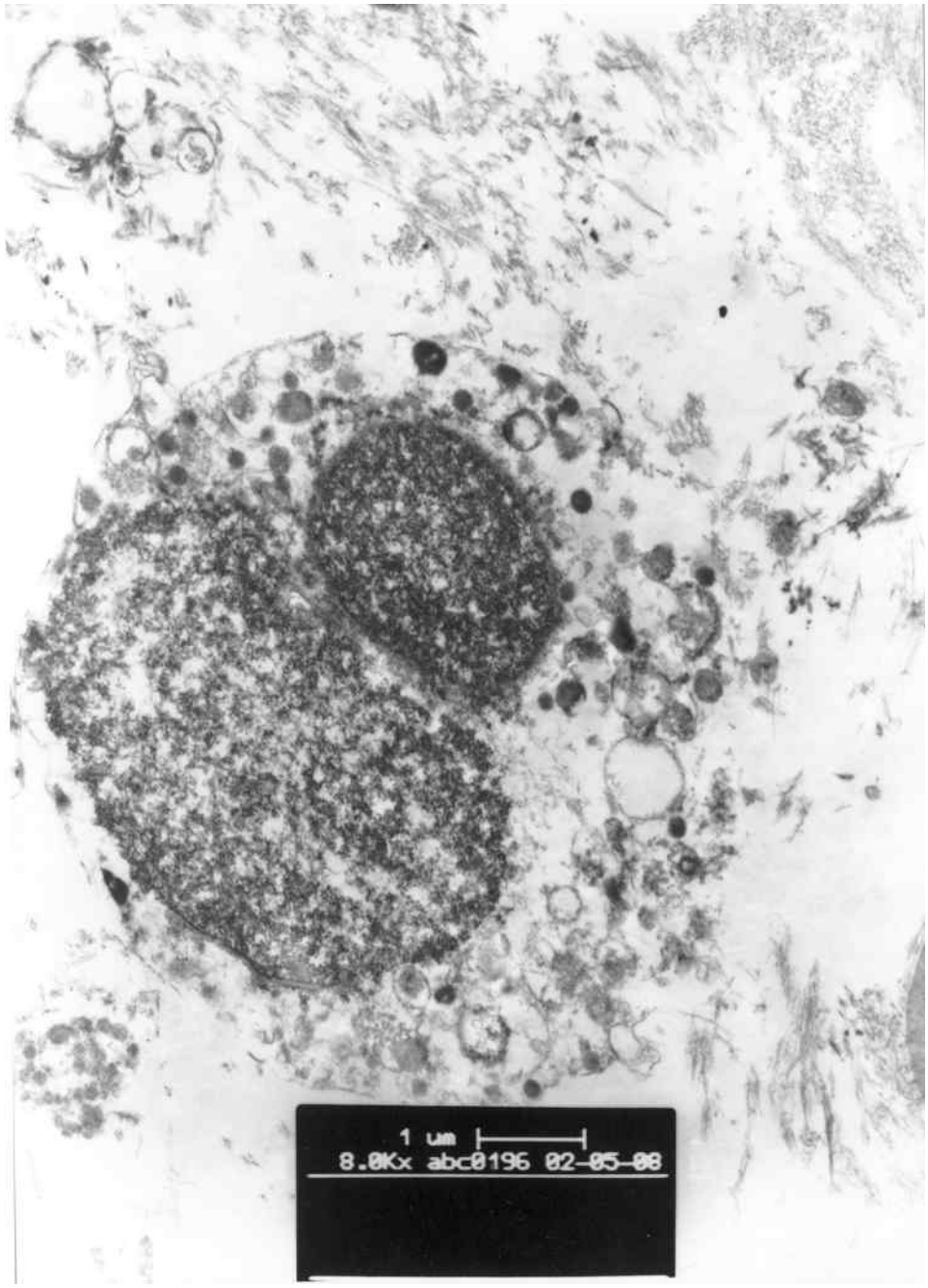

FIG. 4 *Electronmicrograph of exfoliated degenerated malignant urothelial cell in the urine of patient with squamous cell carcinoma. The sample was processed by using the direct conventional EM technique. The cell membrane and the remnant of cellular constituent are not as clearly seen as in the previous photo. ×8,800.*

gelatine capsules by using freshly prepared resin at 60°C for 24 h.

Ultramicrotome (Leica Ultracut R) was used to do ultrathin sections. Double-stained sections with uranyl acetate and lead citrate were done. Examination was performed by using Philips EM 208 S.

Statistical analysis was performed. The student *t* test was used to compare the obtained results.

RESULTS

In this study, many trials were performed with respect to fixation of cell sediment in glutaraldehyde prior to or post agarose gel embedding, fixative concentration, and the optimum time for fixation to get the best results for cell preservation and staining. The best result obtained for cellular preservation and staining was the fixation of urine sediment for 1 h in 4% buffered

glutaraldehyde with sodium cacodylate 0.2 M prior to embedding with melted agarose. Then we refixed the solidified agarose cell block for 2 h in 4% buffered gluteraldehyde. The agarose cell block must be cut into tiny pieces before fixation in glutaraldehyde.

The mean number of urothelial cells detected in Pap stained urine smear of inflammatory cystitis was 5.25 ± 4.72/HPF and 12.36 ± 6.96/HPF in malignant cases versus 7.31 ± 4.07/HPF and 20.6 ± 8.2/HPF, respectively, in the corresponding agarose cell block paraffin prepared section and stained with hematoxylin and eosin stain. This observation was confirmed in electron microscopy prepared samples by agarose cell block versus conventional techniques.

Urine samples processed by using the agarose cell block technique revealed well-preserved cellular constituents, easily identified cell contour and cellular details in viable cells versus the sample prepared by a conventional technique (Figures 1–4).

DISCUSSION

The reliability of urine cytology in the detection of urothelial neoplasm depends on various factors, including tumor size, tumor grade, the quality of the sample preparation method, and experience in interpretation [8]. For effective electron microscopy examination, the material must be representative and must be properly handled and processed [9].

In the present work, the application of agarose cell block technique allowed the cell sediment to be coated with agarose and processed in block manner, so loss of cells during manipulation was avoided. This is in comparison with the samples processed by the direct conventional EM procedure in which during the pouring off the supernatant, after the centrifugation from one step to another, there was definitive loss of cells. This cellular loss was also encountered during the lay of cell sediment in the hole formed in the solidified gelose in agar technique done in this study. Moreover, in the present work there was a significant increase in cell separation in agarose cell block prepared paraffin sections versus the Pap-stained smear of malignant cases. It is expected that some cells are lost during spreading of the sediment on the slide and the sample is often not uniformly distributed. This is in agreement with Farrow [10], who reported that direct smear was easier to prepare but showed scanty cellularity.

The low melting point of agarose gel was an another advantage met during this work. It allowed uniform distribution of cell sediment in the formed block, as the sediment cells were suspended in the melted agarose, which remained in a liquid form during centrifugation. Thus, sedimentation of cells in this supporting media occurred in a proper way. Cellular preservation was evident in cases prepared using agarose cell block technique. Cell membrane was often intact in viable cells and nucleoli were evident by using this technique. These can have great impact on making proper diagnosis.

When we compared agarose embedding media applied in this study with other already used media, such as agar and albumen, we found also some advantage for the former technique. The preparation of cell block using 2% agar as mentioned by Morse [4] is not usually successful, because the agar solidifies before it is well mixed with the sediment and forms fragments. Moreover, in agarose cell block technique, we didn't leave the specimen to be infiltrated with agarose gel for 1 h, as when we used 15% aqueous bovine serum albumen (BSA) as embedding media [3].

In the present technique, simultaneous processing of the same block for light and electron microscopic examination offers a new prospect for cytopathology. The same sample can be simultaneously examined using Pap-stained smear, paraffin agarose cell block sections stained with hematoxylin and eosin, and EM agarose cell block ultrathin sections stained with uranyl acetate and lead citrate. This can aid in the under standing and categorization of atypical urine cytology. Indeed, early detection or prediction of tumor has a great impact on the outcome of proper medical intervention.

Moreover, these paraffin sections can be used to perform tumor markers, which can be of help in diagnosis and follow-up later on. Krestens et al. [5] report that in diagnostic cytology, it has been advocated that molecular techniques will improve cytopathological diagnosis and may predict clinical course.

In conclusion, EM agarose cell block technique is an easy applicable technique, which has many advantages. It is worth white to evaluate its effectiveness as a routine technique in cases of urine cytology and cell suspensions.

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