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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Cell blocks in pleural effusions: comparing agar gel and cell tube methods

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA

Artigo de investigação médica

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2016

Agradecimentos

Nesta etapa do meu percurso académico queria deixar os meus agradecimentos a quem me deu apoio institucional, académico, científico e laboratorial.

Em primeiro lugar o meu sincero agradecimento ao Prof. Doutor Rui Henrique por ter aceitado prontamente orientar este trabalho, pela forma sincera e pragmática como abordou as várias questões que lhe estavam direta ou indiretamente relacionadas e pela sua generosidade em transmitir conhecimentos. Agradeço também a sua total compreensão para aspetos da minha vida familiar e profissional. O Professor Rui Henrique é uma referência para todos os que tiveram o privilégio de serem seus alunos e colegas (tenho testemunhado isso ao longo destes anos no ICBAS e do qual sou exemplo). Da minha parte, espero poder continuar a contar com o seu apoio científico e académico, e acima de tudo ser sempre capaz de retribuir a sua confiança e amizade. Personalizo no Professor Rui Henrique os meus agradecimentos institucionais ao Centro de Investigação do IPO-Porto.

Ao meu coorientador o Prof. Doutor Ricardo Marcos, agradeço o fato de acreditar na minha capacidade para levar a cabo a aplicação da técnica do *cell tube block,* a qual descreveu pela primeira vez em amostras de Medicina Veterinária. Não posso deixar de referir também todo o seu empenho e participação na realização dos procedimentos práticos de obtenção dos *cell tubes*.

À Prof.ª Doutora Carmen Jerónimo agradeço ter autorizado a utilização do laboratório que dirige para a realização desta nova técnica. A Professora Carmen tem uma vasta carreira científico-académica, coordena um grupo entusiasta e dinâmico de investigadores e, por isso, foi para mim um privilégio que tenha aceitado colaborar neste trabalho.

Às Mestrandas Francisca e Sofia do Grupo de Epigenética & Biologia do Cancro agradeço o apoio em questões práticas deste trabalho. Incluo também neste agradecimento os restantes Estudantes de pós-graduação do mesmo grupo, que tiveram o cuidado de avisar sempre que chegava uma nova amostra de derrame pleural.

À Fernanda Silva, Conceição Martins e Paula Lopes do Serviço de Anatomia Patológica do IPO-Porto agradeço a dedicação e mestria para a obtenção, corte, coloração e realização das marcações imunocitoquímicas nos citoblocos.

À Doutora Carla Correia-Gomes do *Scotland's Rural College*, uma amiga e investigadora que tanto admiro, e a quem devo um agradecimento pela ajuda com a análise estatística dos dados.

À Fernanda Malhão do ICBAS-UP agradeço a forma dedicada como procedeu às digitalizações das lâminas dos casos incluídos neste trabalho e, acima de tudo à partilha da sua experiência no processamento dos *cell tubes*. O meu agradecimento ao Laboratório de Histologia e Embriologia do ICBAS, onde trabalho, pela possibilidade de usar o sistema de digitalização de lâminas.

Os meus agradecimentos institucionais ao ICBAS que me acolheu pela primeira vez há quase vinte anos (mais precisamente em setembro de 1997), local onde tenho desenvolvido toda a minha carreira académica, quer como estudante quer como docente, procurando sempre honrar a qualidade que o caracteriza enquanto instituição de ensino e de investigação. Nesta fase específica do percurso académico, personalizo estes meus agradecimentos no Regente desta unidade curricular, o Prof. Doutor Martins da Silva. Aproveito para deixar uma menção e um enorme obrigado ao exemplar trabalho desenvolvido pela Júlia Sousa e Patrícia Soares do secretariado do ensino pré-graduado do ICBAS.

À minha família, em especial a minha Mãe que continua a dar-me uma ajuda insubstituível, cuidando do meu filho Martinho nos dias de mais trabalho…E por fim, o que dizer ao meu Martinho…espero que no futuro consigas perceber o porquê desses dias em que a Mãe trabalhava mais e que vejas em mim o exemplo de alguém dedicado, confiável e esforçado.

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A Dissertação que se apresenta está de acordo com as regras preconizadas pela revista *Diagnostic Cytopathology* para a redação de *Original Articles* (Artigo de Investigação Original).

Abstract

Background: Cytological examination is a cornerstone in the management of cancer patients with effusions. Cell blocks have been increasingly recognized as valuable source for ancillary tests in malignant effusion samples. In this study we investigated the feasibility of a new and simple cell block method, the cell tube block (CTB) in pleural effusion specimens. Additionally, morphologic features and immunocytochemical performance of CTB were compared to a conventional agar cell block (ACB) method (HistoGel™).

Methods*:* A series of 22 pleural effusions from breast and lung cancer patients was studied. For preparing CTB, capillary tubes were filled with the specimen, spun in a micro-hematocrit centrifuge, broken at the liquid-solid interface, and formalin fixed. After paraffin embedding, CTB were cut and routinely stained with H&E and immunostained for pan-cytokeratin, vimentin and Ki67. A comparative evaluation of morphology and immunocytochemistry performance was done in 16 cases.

Results: CTB was straightforwardly performed in all the cases, yielding an overall morphological preservation similar to ACB, with the latter being relatively superior only in nuclear details. No difference was detected between the methods regarding the proportion of positivity for pan-cytokeratin, vimentin and Ki67. The intensity of immunolabelling was also similar, but a significantly higher background occurred in ACB.

Conclusion: CTB method rendered an overall morphological preservation and immunostaining performance comparable to traditional ACB. The simplicity, lower cost and feasibility in blood-rich specimens were advantages of CTB. Further studies are needed to evaluate the suitability of CTB in other cytological specimens and as a platform for assessing genomic biomarkers in cancer patients.

Key Words: cytopathology, pleural effusion, cell block, immunocytochemistry.

Introduction

Since the first diagnosis of cancer made in a body cavity fluid in 19th century, effusion cytology has gained chief importance in clinical and research settings, being nowadays used world widely as a routine diagnostic procedure.[1,2]

Cell blocks correspond to paraffin embedded cellular material obtained from different cytological specimens, including rinses of fine needle aspirations and effusion fluids. Cell block techniques have been described for long and nowadays there are several reports on their use for diagnostic and research purposes.[3] The main goal for preparing cell blocks is to recover and concentrate exfoliated or aspirated cells and thus generate material that can be used for ancillary tests.[4] A myriad of techniques for obtaining cell blocks have been described over the past decades and methods often differ among institutions.[3,5,6]

Cell blocks not only preserve cells but also allow the use of panels of antibodies in multiple consecutive sections, with protocols comparable to those of surgical biopsies.[3,7,8] They also permit archival storage of cytological specimens, such as the surplus body fluids that otherwise would be wasted, thus providing a valuable source for retrospective studies.[9] Moreover, cell block sections can provide a histological and cross-sectional view of cell clusters, such as papillary or duct-like formations (frequently missing in cytological smears), thus helping to establish the histogenesis of the pathological process.[10]

Limitations of cell blocks have also been pointed out, such as the increased turn-around time and the technical complexity of some methods.[3] Effusion fluids contaminated with blood are more laborious, as hemolysis should be attempted prior to cell block preparation.[11] Blood-rich specimens are often collected from patients and present major problems: not only blood can jeopardize immunocytochemical results

due to high background, but also the recovery of cells is more difficult because of hemodilution.[12] Nowadays these drawbacks struggle with the increasing demand to use cytological material for molecular and genetic tests.[13,14] In patients with advanced metastatic diseases, it is nowadays acknowledged that cell blocks represent a unique platform for diagnostic and prognostic purposes.[15,16]

Recently, a new technique to generate cell blocks from effusion fluids (including blood-rich specimens) was described in veterinary medicine specimens.[17] The technique, so-called “cell tube block” (CTB), has been considered to have the same general advantages of cell blocks, with additional benefits of simplicity, low-cost and stratified appearance of the cells in the sections, with separation of blood elements and mesothelial from neoplastic cells.[17] This new technique requires capillary tubes and three simple steps: filing of the capillary tube with liquid, spun in a micro-hematocrit centrifuge and breaking the capillary tube at its liquid-solid interface. Subsequently, the CTB is fixed and routinely processed, similar to a small tissue biopsy.

The aims of this prospective study were: 1) evaluate if the CTB method could be used in human samples — this was assessed in the preliminary evaluation; 2) compare this method with a conventional agar cell block (ACB) method (using HistoGel^{TM}) — this corresponding to the comparative evaluation. Both the preservation of morphological features and immunocytochemical staining was compared between these two methods in pleural effusions from cancer patients.

Materials and Methods

Samples and processing

Twenty-two pleural effusion samples received at the laboratory of the Research Center (CI-IPOP), Portuguese Oncology Institute of Porto, between May and August 2016 were included. These fluids were collected under the scope of a research protocol

approved by the Institutional Review Board (approval number 120/015 at 04/06/16) and a written informed consent was provided by each patient. All these patients (n=20) had a clinical history of cancer: 11 had primary lung malignancy and nine breast tumors (Table I). In all cases the pleural fluid was received refrigerated and it was processed within one hour after the collection. The fluid was firstly divided into 50-ml aliquots and centrifuged for ten minutes at 3076 g at 4ºC (Sigma 3-16PK, Osterode, Germany) (Fig. 1). The supernatant was decanted and the sediment of one aliquot used for performing the CTB.[17] Briefly, plain capillary tubes (Marienfeld, Lauda-Königshofen, Germany) were filled with fluid and tapped with commercial modeling clay (Plastilina Jovi, Rubí, Spain). In non-bloody fluids a high-density solution (Percoll, Sigma-Aldrich, St. Louis, USA) was used to ensure a proper separation from the clay. In this case, after filling the tube with the sample, an air bubble was introduced by gently rocking the tube and then 5-10 µl of the high-density solution was sucked up by capillarity. In all the cases, the tubes were spun in a micro-hematocrit centrifuge (Sigma 201m) at 12000 g for five minutes, and tubes were cut at the liquid–solid interface with a glass writing-diamond pen. The CTB was fixed in 10% formalin for 24 hours and then routinely processed for paraffin embedding using an automated tissue processor under standard conditions for surgical biopsies. A specific step occurred at embedding, since the CTB was removed from the (remaining) capillary tube using a modified paper clip (Fig. 1). In cases with a large brown area of red blood cells or with a Percoll™ area, those areas were separated from the remaining pellet with a surgical blade before embedding.

To construct the ACB, the sediment from seven 50-ml aliquots was used. Whenever the cell pellet was bloody, hemolysis was performed by resuspending the cell pellet in ethylenediaminetetraacetic acid (EDTA) solution with NH_4Cl and $KHCO_3$ for 30 minutes in ice. Then, the tubes were again centrifuged for ten minutes at 3076 g, at 4ºC. After decanting the supernatant, the cell pellet was resuspended in phosphate buffered saline, transferred to a 1.5 ml tube and centrifuged for another ten minutes at 9391 g at 4ºC. Supernatant was poured off and the pellet frozen at -80ºC (for biobanking

purposes). After a sufficient number of cases (at least ten) have been gathered, the remaining ACB protocol followed. Cell pellets were thawed at room temperature, resuspended in 2 ml of 96% ethanol and transferred to 15-ml centrifuge tube. The mixture was centrifuged for ten minutes at 1335 g (Sigma 4-15 Sartorius). The supernatant was discarded and melted commercial agar (HistoGel™, Thermo Scientific, Waltham, USA) was added. After another centrifugation for ten minutes at 1335 g, the mixture was cooled at -20ºC for ten minutes. The mixture was carefully taken out of the centrifuge tubes with a stick, the conical cell pellet was sectioned in half and placed in a histological cassette. After 24 hours in 10% formalin, it was routinely processed. The same routine automated processing, as well as sectioning (3 µm) and hematoxylin-eosin (H&E) staining was applied to CTB and ACB.

Immunocytochemistry

Immunocytochemistry (ICC) was performed simultaneously on CTB and ACB sections using routine protocols for formalin-fixed, paraffin embedded tissues, including appropriate positive and negative controls. The ICC panel included commonly used antibodies against intermediate filaments [pan-cytokeratin (NCL-L-AE1/AE3 clone, Leica Biosystems, Newcastle, UK), dilution 1:250]; vimentin (NCL-L-Vim-V9, clone V9, Leica Biosystems, Newcastle, UK), dilution 1:100], and a nuclear marker [Ki67 antigen (Ki 67-M7240, MIB-1 clone, Dako, Glostrup, Denmark), dilution 1:200]. The equipment Leica Bond III and the Bond Polymer Refine Detection system (both from Leica Biosystems) were used for pan-cytokeratin and vimentin detection, while the MIB-1 antibody labeling was performed in the Benchmark Ultra Ventana equipment using the Ultra View Universal DAB Detection Kit (both from Ventana Medical Systems, Tucson, USA).

Comparative evaluation

Routine H&E slides of CTB and ACB were qualitatively assessed for cell distribution within the section, type of cells, identification of malignant cells, and separation of red blood cells from the other cells elements and presence of artifacts. Additionally, slides digitalized with a 20x objective (Virtual Slide Microscopy, VS110, Olympus,Tokyo, Japan) allowed for a blinded comparison of cell morphology preservation. Two similar areas were selected in each section, and digital images reviewed blindly to the cell block method by an experienced pathologist, using the OlyVIA Software version 2.4 (Olympus). A three-point scoring system (1 – poor; 2 – moderate; 3 – good) was used and five morphological parameters assessed: 1) presence of cell groups and maintenance of architecture; 2) definition of cytoplasmic limits; 3) definition of nuclear membrane; 4) preservation of chromatin pattern; 5) identification of nucleoli. As to the ICC evaluation, the positivity (herein defined as at least one cell with the expected staining), the pattern (surface, cytoplasmic or nuclear), the intensity and the background were evaluated by a consensus of two observers in a multi-headed microscope. For assessing intensity and background staining, the three-point scoring system (1 – absent or low; 2 – medium; 3 – high) was used. For Ki67 only positive *versus* negative assessment was performed (positivity defined as one or more cells with nuclear staining).

Statistical analysis

Statistical analysis was performed with R, version 2.12.1 from R Foundation for Statistical Computing (http://www.r_project.org). Differences between scores of morphological and immunocytochemistry parameters were assessed using the Wilcoxon signed-rank test, with Bonferroni's correction (statistical significance set at $p < 0.05$). The agreement between the two cell block methods was also assessed with kappa (k) statistics. For interpreting the strength of agreement, the following standards were considered: ≤ 0.40 = poor, 0.41-0.60 = moderate, 0.61-0.80 = good and 0.81-1 = almost perfect.[18] Additionally, the level of overall concordance (*i.e.,* positive *versus*

negative staining for each antibody) in slides from the two methods was also assessed. Regarding Ki67, a McNemar's test was used to compare paired proportions of positive cases in CTB and ACB.

Results

Twenty-two pleural effusions specimens were obtained from 20 cancer patients (seven male patients and 13 female patients) with a mean age of 60 years (range: 28 to 83 years-old). Nine patients had been previously diagnosed with breast cancer, eight patients had lung adenocarcinoma as primary malignancy, whereas two patients presented small cell lung carcinoma, and one patient had lung squamous cell carcinoma (Table I).

Preliminary evaluation

The CTB procedures were straightforward, even if technical issues occurred during sectioning. We identified fixation and embedding steps as two pre-analytical factors that could affect the quality of the sections. Regarding the former, cell pellets exceeding 6 to 8 mm showed poor fixation in the area closer to the modeling clay; this was managed in subsequent cases by filling capillary tubes with variable amounts of fluid. As to embedding, cooling down the broken capillary tubes helped the removal of CTB pellet; however, we verified that a direct contact of the pellet with moistened surfaces could partially hydrate paraffin resulting in sectioning problems.

In cases presenting a mixed cell population composed by red blood cells, inflammatory, mesothelial and neoplastic cells, a stratified arrangement of the cells was often noticeable, with the following sequence (starting from the clay): red blood cells, inflammatory plus mesothelial cells and then groups of neoplastic cells (near the liquid-solid interface) (Fig. 2).

Comparative study

A comparative evaluation was performed in 16 paired CTB and ACB. Neoplastic cells were found in the same 11 cases (69%) in CTB and ACB sections. In the remaining 5 cases (one patient with small cell lung carcinoma, one with lung squamous cell carcinoma, and three with breast ductal carcinomas) inflammatory (mainly lymphocytes) and benign mesothelial cells were observed. In six cases positive for malignant cells in CTB, cell groups with acinar and/or duct-like structures were observed. This was also noted in ACB sections, but in three cases the definition and cohesiveness of the cell groups were less evident.

In ten out of 16 CTB (62.5%), a homogenous distribution of the cells within the section (with similar cell density in high power fields) was observed. In ten out of 16 (62.5%) ACB sections a variable distribution of cells within the section was noted, with densely cellular areas admixed with paucicellular ones. A dark-brown pigment (compatible with a hematein pigment) was frequently observed in CTB (eight out of 16 cases), namely in the part of the section closer to red blood cells. As to ACB slides, this type of pigment was never observed, but three artifacts were identified: 1) eosinophilic fibrin strands with trapped cells (in five cases); 2) eosinophilic proteinaceous lakes, (in 11 cases); 3) basophilic aggregates, presumptively of nuclear debris (in 14 out of 16 cases). In CTB sections this type of basophilic aggregates were never observed, while a small amount of eosinophilic fibrin material was depicted in four cases. As eosinophilic materials were randomly distributed within the ACB sections, they did not hamper the identification of the surrounding cells. In CTB, fibrinous material was unifocal in all the cases. Additionally, areas of poor cellular preservation, denoted by a bleaching of nuclear and cytoplasmic staining were noted in five ACB cases.

Overall, no differences were observed in cell preservation between the two cell block methods (p = 0.18) (Table II). However, the methods presented poor concordance (k <

0.40) regarding all the morphological parameters, except for the presence of cell groups and maintenance of architecture, which were moderately concordant [k = 0.43; 95% confidence interval (0.11-0.74)]. Considering each morphological parameter, ACB was significantly superior for assessing nuclear membrane definition (p = 0.03), and a slight improvement in chromatin detail (p = 0.048) and nucleoli identification (p = 0.049) was also noted.

In the comparative assessment of ICC in the two methods, we verified that sections of CTB were less resistant to the imunostaining steps, presenting a more irregular surface; in all cases, however, representative material (comparable to that of the respective H&E slide) was observed.

Concerning immunomarking, pan-cytokeratin and vimentin showed an overall concordance of 100% in positivity between the methods. The staining was exclusively cytoplasmic, with similar intensity in CTB and ACB slides (Table II). Nevertheless, the areas with poor cellular preservation noted in H&E slides of some ACB were also characterized by a decreased intensity in vimentin labeling. A higher background unspecific staining was observed in ACB, either with pan-cytokeratin or with vimentin (p = 0.02 and p = 0.01, respectively) (Fig. 3). In ACB, moderate background (score 2) was noted in seven and six cases with pan-cytokeratin and vimentin, respectively, whereas in CTB two and seven cases presented such background. High background staining (score 3) was never observed with CTB, but was detected in three and five cases with pan-cytokeratin and vimentin, respectively in ACB. As to Ki67, the overall concordance between the two methods was lower (73%), but no difference in the proportion of positive cases in each cell block method was detected (p = 0.6). No background staining was observed with this antibody in either method.

Discussion

Cytopathological examination of body fluids is of paramount importance for diagnosis and staging of cancer patients. Currently, it is acknowledged that cell blocks prepared from residual effusion specimens represent an additional and reliable tool, not only for diagnostic purposes, but also for further ancillary tests and research studies.[2] Over the years, several cell blocks techniques have been described and evaluated, and all present advantages and disadvantages.[3]

In this study, a new and simple cell block method, previously described for veterinary specimens,[17] was evaluated in 22 pleural effusions from patients with breast and lung cancer. Firstly, in the preliminary assessment, we demonstrated that CTB could be successfully applied to human pleural effusion samples and the technical issues identified at that stage were easily solved. Despite the diagnostic yield of this new cell block method was out of the scope of the present study, malignant cells were identified in the same cases in CTB and ACB. This is in accordance with previous studies which demonstrated that independently of technique, the diagnostic accuracy of cell block routine slides is not significantly different, being relatively similar to that of the conventional cytology smears.[3,19] In this vein, the usefulness of the cell block lies more in the availability of diagnostic material for immunocytochemistry and molecular tests,[2] and the technique should be viewed as complementary, but not as a substitute of cytological examination.[8]

In the comparative evaluation, we concluded that CTB granted an overall morphological preservation similar to ACB. Still, nuclear details (membrane, chromatin and nucleoli) were better preserved in ACB than in CTB. Probably, formalin fixation of CTB (contrasting with alcohol pre-fixation in ACB), might account for such difference, since formalin is considered the least satisfactory fixative for preserving nuclear details from a cytologist's perspective.[3,19] It is worthy of note that the lesser morphological

definition of nuclei in CTB did not jeopardize the identification of cell types. Moreover, as above mentioned, cell blocks should not replace the prior evaluation of conventional cytological preparations, which are characterized by an excellent preservation of nuclear features.[3,8,20]

ICC performance was also similar with both methods. In this regard, formalin is considered the universal fixative[3] and some authors have warned that a methodological validation is needed for alcohol fixed samples, especially when nuclear epitopes are considered.[21,22] The use of ethanol previous to formalin fixation for preparing ACB is not mandatory (fixation with formalin only has been reported), but it was used in our protocol as it granted a more solid pellet with easier handling (data not shown). Even if overall performance was similar, the two methods significantly differed in background staining. The presence of background staining in ACB has been already described,[23] and this may be due to several reasons: proteinaceous nature of the majority of effusion fluids,[7,24] existing cellular debris (including those resulting from the lysis of red blood cells, as well as those resulting from the deep-freezing of pellets in our protocol) and some immunogenicity of the agar material trapped between cells. It should be stressed that CTB never produced high background and this was probably due to high speed centrifugation that created a liquid-solid interface, assuring that most of the proteinaceous fluid was separated from the cell pellet.[17]

The CTB procedure had several general advantages: it was simple, required no special or expensive equipment (micro-hematocrit centrifuges still exist in most hospitals and capillary tubes are inexpensive) and could be easily learned (inexperienced operators performed all the steps after a short training). Moreover, a separation of cellular elements from diagnostic malignant cells was usually achieved and red blood cells were separated from the cell pellet, thus obviating the need for a hemolysis step that is included in other cell blocks techniques. In this vein, the CTB is particularly useful for

effusions containing large amounts of blood or inflammatory cells. It is noteworthy that some of those advantages have been reported with other alternative methods, such as the cotton block method.[12] On the other hand, ACB method was a more tedious process, with several centrifugation steps and with a special need for converting and maintaining the commercial agar in a liquid state.[3,23] A main hurdle during for any cell block procedure is to avoid cell loss during processing.[4] HistoGel™ and other adjuvants have been introduced to harden the cell pellet, so that it can be easily transferred to cassettes without losing any diagnostic material.[3] In CTB this critical step is surpassed by the use of capillary tubes, which avoid the loss of material during automatic processing.

Despite the encouraging results, some limitations of the present study should be pointed out. Firstly, the number of analyzed cases is limited and restricted to pleural effusions and the ICC panel was narrow, with only three markers. It should be noted that we did not attempt to perform a proliferative index or an interpretation based on a cutoff point for Ki67, as it might be indicated for prognostic or predictive purposes. We thus plan to continue this study and test well acknowledged prognostic/predictive biomarkers for breast and lung cancer. Such an approach would be mandatory to evaluate the utility of CTB in the management of patients presenting at advanced stage disease, as previously done for other cell block techniques in other types of samples.[16,25,26] Additionally, we intend to extend our sample by including cases submitted to cytological diagnosis; this will allow us to evaluate the usefulness of CTB for different samples (such as fine needle aspiration rinses, other body cavity fluid such as peritoneal washings) in a clinicopathological diagnostic context. Moreover, the suitability of CTB for preparing cell microarrays — a tool for a rapid screening of tumor markers in a large number of samples[27-29] — deserves further studies. Recently, Hu et al[29] developed a new procedure for obtaining cell cylinders from pleural effusion samples for cell microarray construction. We hypothesize that the typical cylindrical

shape of CTB, with separation of malignant cell groups from blood and inflammatory cells, could be suitable for cell microarray construction.

In conclusion, CTB method rendered morphological preservation and immunostaining performance comparable to that of traditional ACB method. The simplicity, low cost and feasibility in blood-rich specimens were advantages of CTB that should prompt a large scale study for assessing the use of this methodology in other cytological specimens used for diagnosis. Additionally, the efficacy of CTB as a platform for the assessment of other prognostic or predictive cancer biomarkers, including genomic-based, deserves further investigation.

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Figure legends

Fig. 1. Cell tube block (CTB) methodology. Pleural effusions were sent to the laboratory immediately after collection (A), divided in 50-ml aliquots and centrifuged for 10 minutes at 3076 g at 4ºC (B); after pouring off the supernatant, the sediment of one aliquot (C) was used to fill capillary tubes, which were spun in a micro-hematocrit centrifuge at 12000 g for five minutes (D), resulting in cylindrical cell pellets — CTBs. When the CTB was too close to the clay, an air bubble and a small amount of Percoll^{TM} (asterisk) was inserted, thus assuring that the CTB was easily individualized from the clay (F, on the left, capillary tubes before and after centrifugation, and on the right, the same case before and after centrifugation, using Percoll^{TM}). After breaking capillary tubes at the liquid-solid interface, the tubes were fixed in formalin for 24 hours (G) [detail of the CTB after fixation (H)] and routinely processed within a traditional cassette (I). Before paraffin embedding, the CTB was pulled away from the remaining capillary tube using a paper clip (J, K). Final appearance of paraffin blocks of CTB (L).

Fig. 2. Representative cell tube block sections from a pleural effusion of a metastatic ductal breast carcinoma (case 11). A stratified arrangement was noticeable in H&E (A) and highlighted by the immunostaining for pan-cytokeratin (B) and vimentin (C). Starting from the clay, the following sequence appeared (right to left in the inset): red blood cells, inflammatory plus mesothelial cells (vimentin positive and some also positive for pan-cytokeratin, asterisk) and, then groups of pan-cytokeratin positive, vimentin negative adenocarcinoma cells (with acinar and ductal-like structures) up until the liquid-solid interface (arrow). Bar= 140 µm (100 µm inset)

Fig. 3. Paired cell tube block (CTB) and agar cell block (ACB) (HistoGel^{TM}) of a lung adenocarcinoma (case 15). In H&E (A, B), individualized malignant adenocarcinoma cells were observed in CTB near the liquid-solid interface (arrows, A) and were also

identified in ACB (arrow, B), scattered among inflammatory and mesothelial cells; in this case, an eosinophilic proteinaceous material was also observed (arrowheads). With pan-cytokeratin (C, D), malignant cells exhibited intense cytoplasmic staining, both in CTB (C) and ACB (D) and no background staining was detected in this case. As to vimentin (E, F), it marked inflammatory cells (adenocarcinoma cells were negative, arrows) in CTB (E) and ACB (F), but in this latter a moderate background staining was visible. Ki67 immunolabeliling (G, H) stained nuclei similarly in CTB (G) and ACB (H). Bar = 65 µm.

Tables

Table I. Clinicopathological data of pleural effusions cases used for the preliminary and comparative studies (ns: not specified).

Case	Age (years)	Sex	Primary malignancy
Preliminary study			
1	28	M	Lung adenocarcinoma
2	28	M	Lung adenocarcinoma
3	49	M	Lung adenocarcinoma
4	58	M	Small cell lung carcinoma
5	62	F	Breast carcinoma (ductal subtype)
6	55	F	Lung adenocarcinoma
Comparative study			
7	61	F	Breast carcinoma (ns)
8	55	F	Breast carcinoma (mixed ductal/micropapillary subtype)
9	68	F	Breast carcinoma (ns)
10	58	M	Lung adenocarcinoma
11	50	F	Breast carcinoma (ductal subtype)
12	51	F	Breast carcinoma (ductal subtype)
13	62	F	Breast carcinoma (ductal subtype)
14	50	F	Breast carcinoma (ductal subtype)
15	72	M	Lung adenocarcinoma
16	83	M	Small cell lung carcinoma
17	56	F	Breast carcinoma (ductal subtype)
18	62	F	Lung adenocarcinoma
19	50	F	Lung adenocarcinoma
20	79	F	Lung adenocarcinoma
21	55	F	Lung adenocarcinoma
22	79	M	Lung squamous cell carcinoma

Table II. Average score of morphological parameters and immunocytochemistry results on cell tube and agar gel (Histogel^{TM}) blocks in 16 effusions from cancer patients. A three-point scoring system (1 – poor/low or absent; 2 – moderate/medium; 3 – good/high) was used for each parameter, except for Ki67 (positive *versus* negative evaluation); ns: non-significant.

	Cell tube block	**Agar gel block**	***p value***
Morphology			
Architecture/cell groups	1.9	2.0	ns
Cytoplasmic limits	1.4	1.3	ns
Nuclear membrane definition	2.1	2.5	0.03
Chromatin pattern preservation	1.8	2.3	0.048
Nucleoli identification	1.9	2.4	0.049
Immunocytochemistry			
Pan-cytokeratin intensity	2.5	2.9	ns
Pan-cytokeratin background	1.1	1.9	0.02
Vimentin intensity	2.9	2.5	ns
Vimentin background	1.5	2.1	0.01
Ki67 positivity (%)	93	80	ns

FIGURE 1

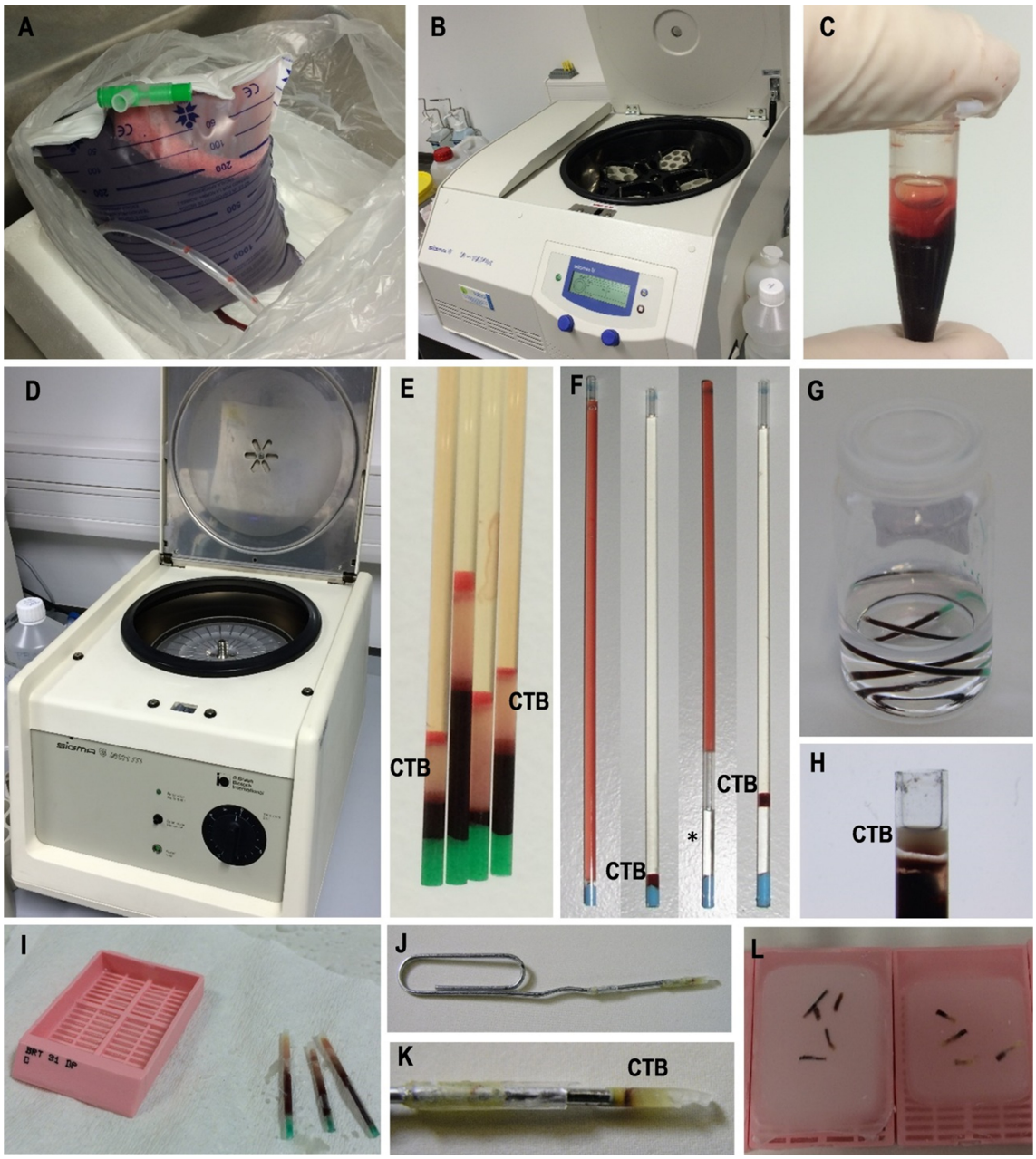


FIGURE 2

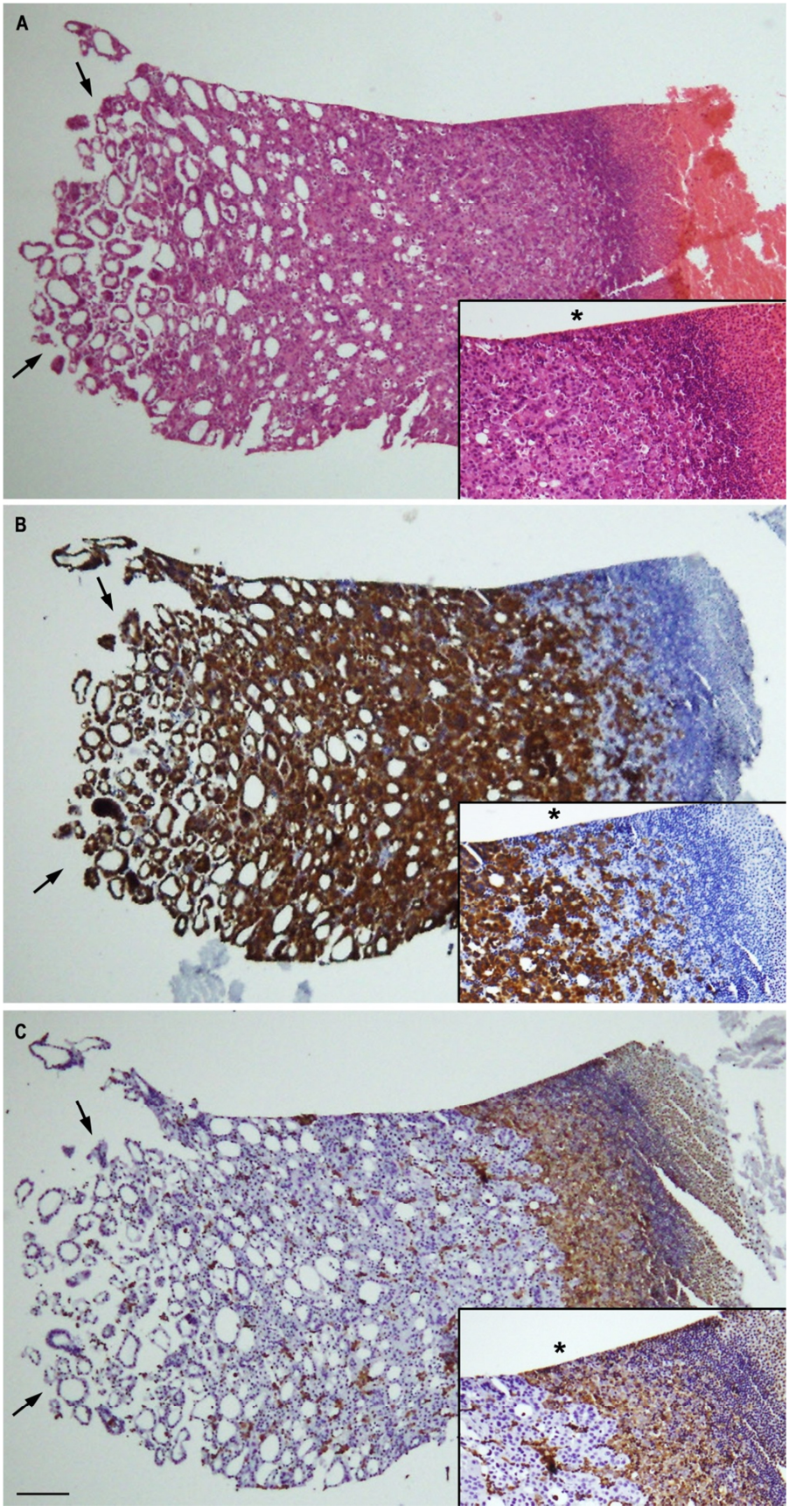
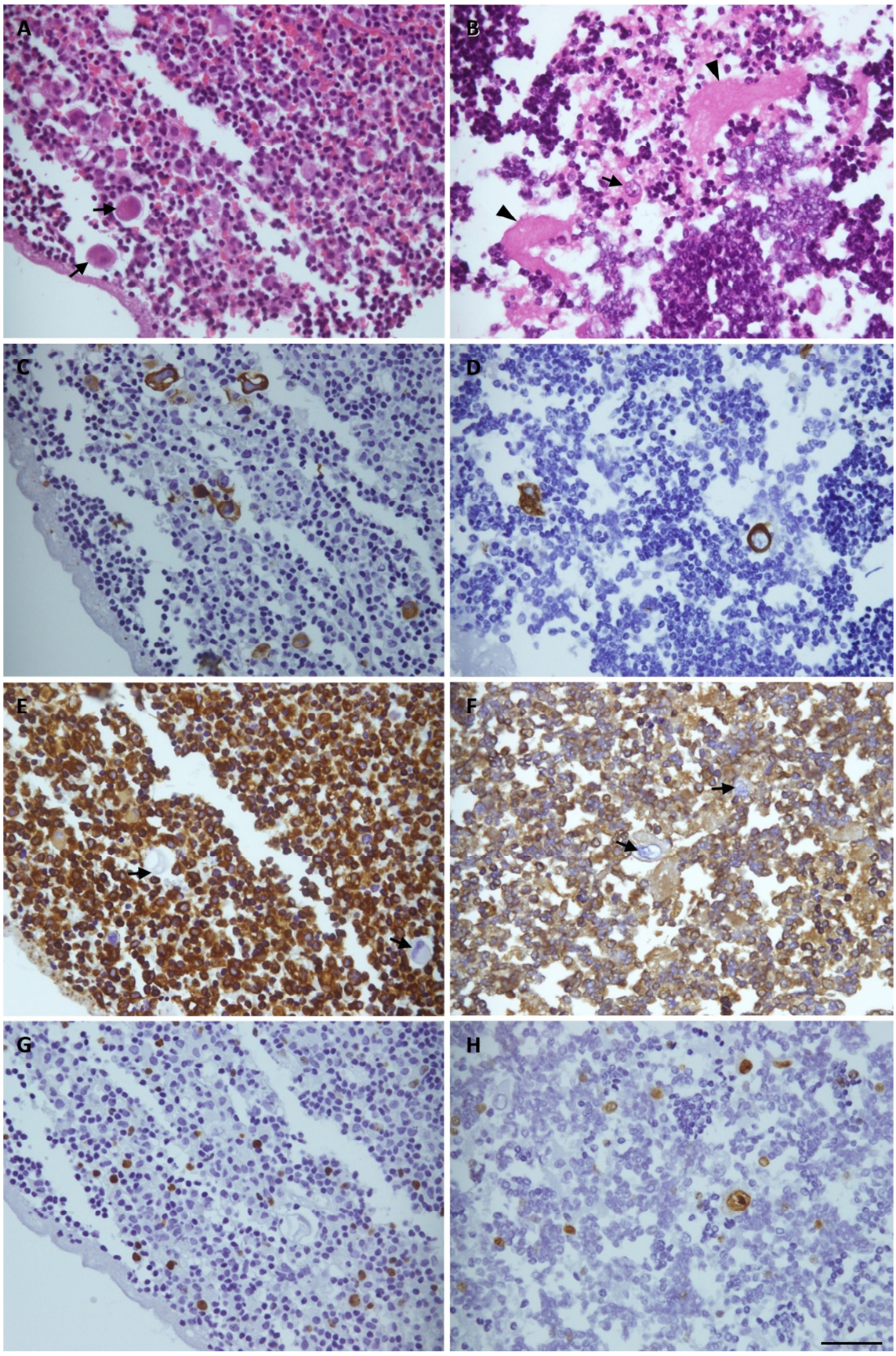


FIGURE 3



Citoblocos em derrames pleurais: comparação dos métodos agar gel e citotubo

Resumo

Introdução

Na atualidade, a Citopatologia desempenha um papel insubstituível na avaliação dos derrames cavitários. Esta tem sido complementada pelo uso dos citoblocos, que permitem a realização de técnicas de deteção de biomarcadores, como seja a imunocitoquímica. Desde da introdução dos citoblocos no diagnóstico, diversas metodologias para a sua obtenção têm vindo a ser descritas, cada uma com as suas vantagens e desvantagens. Recentemente foi descrita uma nova técnica de preparação de citoblocos, designada citotubo, a qual foi testada em amostras obtidas na prática clínica veterinária. Esta técnica inclui procedimentos simples que passam pela colocação das amostras em tubos de microhematócrito, posteriormente centrifugados para compactar as células. Os tubos são subsequentemente cortados na interface sólido-liquido, fixados em formol e sujeitos a um processamento de rotina para inclusão em parafina, em tudo semelhante ao de qualquer material de biopsia histopatológica.

Neste contexto, os objetivos do presente estudo foram: 1) avaliar a exequibilidade da técnica de citotubo em amostras de derrames pleurais no âmbito da medicina humana; 2) efetuar uma avaliação comparativa relativamente à preservação da morfologia celular e à imunomarcação da técnica de citotubo e de uma técnica convencional de citobloco, neste caso usando agar comercial (HistoGel®).

Material e Métodos

Foram incluídos neste estudo 22 derrames pleurais de doentes oncológicos, com carcinoma da mama ou carcinoma pulmonar, previamente diagnosticados. As amostras estavam incluídas num projeto de investigação anteriormente aprovado pela Comissão de Ética para a Saúde do Instituto Português de Oncologia do Porto e foram obtidas mediante consentimento informado dos doentes. Em todos os casos procedeu-se à realização do procedimento do citotubo e em 16 casos foi realizado o estudo comparativo entre o citotubo e o citobloco com HistoGel®. Para o estudo comparativo

foram usadas preparações coradas com hematoxilina-eosina e estudo imunocitoquímico, usando anticorpos anti-pancitoqueratina, anti-vimentina e anti-Ki67. No estudo comparativo foi usado um sistema de classificação numa escala de 1 a 3 (1 – baixa; 2 – moderada; 3 – boa), tendo-se avaliado os seguintes critérios morfológicos: 1) presença de grupos celulares/preservação da arquitetura; 2) definição dos limites citoplasmáticos; 3) definição da membrana nuclear; 4) identificação do padrão de cromatina; 5) identificação dos nucléolos. Relativamente à imunocitoquímica avaliou-se a imunorreatividade aos anticorpos e a presença de marcação de fundo. Adicionalmente, foi analisada a intensidade de marcação para a pancitoqueratina e vimentina. Para avaliar a intensidade e marcação de fundo foi também usada uma escala de 1 a 3 (1 – ausente/fraca; 2 – moderada; 3 – elevada). Na análise estatística foram usados os testes de Wilcoxon, de McNemar e estatística Kappa, tendo-se fixado como nível de significância o valor de 0,05.

Resultados

O citotubo foi obtido com sucesso a partir das amostras de derrames pleurais, tendo-se identificado, numa fase preliminar, apenas problemas de fixação e inclusão, os quais foram solucionados no decorrer do estudo. Nos casos caraterizados pela presença de uma população mista de células inflamatórias, mesoteliais e grupos de células epiteliais malignas, verificou-se uma estratificação das células nos cortes de citotubo, com as células inflamatórias e mesoteliais mais próximas dos eritrócitos e as células neoplásicas na proximidade da interface sólido-líquido. Foram identificadas células epiteliais neoplásicas nos mesmos casos de citotubo e citobloco com HistoGel®, não se detetando diferenças entre os métodos relativamente aos tipos celulares presentes em cada caso. No estudo comparativo, também não se observaram diferenças globais entre os dois métodos na preservação celular. Ainda assim, os cortes de HistoGel® apresentavam melhor preservação da membrana nuclear e a identificação do padrão de cromatina e nucléolos era mais clara.

Relativamente à imunocitoquímica, não foram identificadas diferenças estatisticamente significativas entre os métodos relativamente à positividade e intensidade de marcação para a pancitoqueratina e vimentina. Do mesmo modo, não se verificaram diferenças na proporção de casos positivos para o Ki67. Contudo, detetou-se um aumento significativo de marcação de fundo com os anticorpos anti-pancitoqueratina e anti-vimentina no método de citobloco com agar.

Discussão

A avaliação citológica de efusões é um dos pilares do diagnóstico e estadiamento de doentes oncológicos. Recentemente, tem-se assistido a um uso e interesse crescentes nos citoblocos como ferramentas complementares à observação citológica.

Neste estudo descreveu-se a aplicação de uma nova técnica de preparação de citoblocos, designada citotubo, a efusões pleurais de doentes oncológicos — esta técnica tinha sido descrita previamente apenas no âmbito da Medicina Veterinária. Paralelamente, comparou-se a preservação morfológica e os resultados de imunocitoquímica no citotubo e no citobloco obtido com agar (HistoGel®). O citotubo foi aplicado com sucesso aos derrames pleurais de doentes oncológicos, conduziu a uma preservação morfológica e imunomarcação sobreponíveis às observadas no citobloco convencional com agar. A esta evidência acrescem outras vantagens do citotubo, como a simplicidade do procedimento, baixo custo, a exequibilidade em amostras com grande quantidade de sangue, sem necessidade de hemólise prévia, assim como a separação das células neoplásicas dos restantes elementos celulares. Face aos bons resultados obtidos, pretende-se alargar o estudo testando a aplicabilidade da técnica de citotubo a outro tipo de amostras citológicas enviadas para diagnóstico citológico, como punções aspirativas ou lavagens peritoneais. Por outro lado, parece pertinente avaliar a eficácia do citotubo como plataforma para a determinação de marcadores prognósticos e preditivos, incluindo os de natureza genética, em diversos tipos de cancro.