Importance of Scanning Electron Microscope study of paraffin-embedded samples for diagnosis purposes

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November 2016

***Abstract*—The diagnosis performed in Pathological Anatomy (PA) is based on the use of light microscopy (LM) to enable the visualization of cellular changes. However, not all of the cellular changes are visible using LM, either due to its limited image resolution, either due to the type of contrast that can be obtained by LM.**

Scanning or transmission electron microscopy (SEM and TEM) are microscopy techniques that can complement the PA diagnosis. Moreover, the information gathered from different types of microscopy may also hold a more complete diagnosis. This complementary information obtained with different microscopy techniques is called "correlative microscopy".

In this study, we made comparative observations between different biological samples in histological sections by LM and SEM. The sample preparations protocol (thickness, drying and conductivity of the section) has been optimized.

The best results were obtained with the following protocol: histological sections of 10 micrometres thickness, dried with T-butanol and increased of the conductivity with metallic coating. The application of this optimized protocol showed that there is a need for a new protocol optimization in some tissues so that the information can be used in diagnosis.

This study shows that the use of the SEM in the correlative microscopy is executable and provides additional useful information to the diagnosis of PA when compared to LM, although, the preparation of protocols is depending on the type of tissue that is used and it is important that they are always optimized.

***Keywords;* Correlative Microscopy, Diagnosis, Paraffin-embedded samples, Scanning Electron Microscopy**

I. Introduction

THE diagnosis elaborated by Pathological Anatomy is obtained using light microscope. However, there are instances where is not always possible to diagnose through LM all of the cellular alterations present in the samples. The light microscope is an instrument that uses visible light to produce a magnified sample image which is then visualized in an imaging system. The sample is placed before the focal point of the objective lens and the image is then magnified by the ocular lens, to a plane convenient to the user [1]–[4]. For the visualization of samples in this equipment, they must be optically transparent, as for example, a thin section of biological tissue. By using different stains, it is possible to reveal certain components of the cell, because stains (*i.e.* sets of chemicals that absorb light at different wavelengths) have affinity for specific areas of the sample [1].The routine stain in light microscopy for the diagnosis in PA is Hematoxylin-Eosin (HE), because as it is a topographic staining, which means, it distinguishes the cell's nucleus from the cytoplasm. The nuclei are purple by Hematoxylin and the cytoplasm is pink by Eosin [5].

Electron microscopy uses a source of radiation other than light. In this case, an electron beam is used to interact with the sample. In the Scanning Electron Microscope, a highly focused electron beam scans the surface of the samples point by point, line by line. The secondary or backscattered electrons in the sample are detected by electron detectors. This signal is then treated in order to create a pixel-by-pixel image on a screen [6], [7].

The formation of the image in the SEM is dependent on the acquisition of the signals produced from the interaction of the electron beam with the sample. The existence of detectors of the various signals emitted is what allows the visualization of the images. This interaction occurs within a volume of excitation on the surface.

There are two types of scattering processes: elastic and inelastic processes. Elastic processes are those in which electrons retain all their energy, resulting in the production of backscattered electrons as the electrons travel back to the surface of the sample and escape into the vacuum. The inelastic processes are those in which the electrons lose energy

and eventually excite the structure of the samples. When these low-energy electrons escape into the vacuum, they are called secondary electrons [8].

Secondary electrons are produced by the interactions between the energetic electrons of the beam and the weak bonding electrons of the metals used in the coating of the sample. If the electrons have enough energy, there will be ionization of atoms. During this phenomenon there is the ejection of electrons that have a certain kinetic energy and reach the detector [9]. In practice, there is generally some asymmetry due to the fact that the secondary electron detector is located to one side of the chamber. Surface characteristics that are tilted toward the detector appear brighter because the electrons emitted by these regions are more likely to reach the detector. Secondary electrons are mainly used for topographic contrast, *i.e.* for visualization of the texture and roughness of the sample. The visualization of a topographic image depends on the number of electrons that reach the detector [8].

Backscattered electrons are electrons from the beam that are projected from a sample by elastic scattering, leaving the sample with energy close to the energy of the primary beam. Thus, they can be distinguished from the secondary electrons by using their kinetic energy [1], [9], [10].

The contrast in the images obtained through the backscattered electrons translates to variations in the atomic composition of the sample. The areas with higher atomic number elements appear lighter in this type of image [1], [9], [10].

X-ray energy dispersion spectroscopy is a qualitative and quantitative microanalytical technique that can provide information on the chemical composition of elements[11].

The SEM allows the visualization of the sample's superficies, creating a three-dimensional appearance. However, it does not have a high resolution unlike the Transmission Electron Microscope (TEM) [12]. Field Emission Scanning (FESEM) is a microscope similar to conventional SEM, with a different electron source, making use of a field emission electron gun [13].

The application of different types of microscopies to obtain different information from the same samples is called Correlative Microscopy (CM) [14]. The same sample is observed trough de different types of microscopies to posterior analysis of the information gathered. The correlation of this information allows a more complete study of the sample [15]–[18]. To use CM, any combination of two or more of the imaging systems that use light, electrons, X-rays or even magnetic resonance imaging are possible [19], [20]. However, the most common use of CM is found in the combination of visible light microscopy and electron microscopy - correlative light and electronic microscopy (CLEM). The combination of these two methods allows the visualization of the cell to find a rare event in a tissue and to identify the local for later visualization in high resolution with electron microscopy [16]–[18], [20]–[23].

The use of CLEM with separate instruments has several obstacles that need to be overcome: recognition and localization of the region of interest in both types of microscopy and observation of the sample in the two microscopes [24]. Thus, the easiest way to correlate is to use the information obtained after the application of different protocols to similar samples, not the same sample, that is, parallel samples that were not prepared in the same way [18], [25].

To perform some diagnoses, CM is used as a combination of (transmission) light microscopy and electron microscopy of samples that may or may not be exactly the same [26]. When the sample is exactly the same, the area of interest is removed from the paraffin to be embedded in resin. After this process, the semi-thin sections of resin sample are analysed to again analyse the area in LM, in order to locate the region of interest and then perform the ultrafine sections for electron microscopy [19].

Although light transmission microscopy and transmission electron microscopy are the most commonly used methods - performing sections of the samples to see under the microscope - there are differences between them in terms of resolution and the area of observation [27]. In order to overcome these differences, the SEM can be used, since it has a resolution range that varies almost from the resolution of LM to that of TEM, in addition to presenting a different image type than that obtained by transmission microscopy, because it gives information about the topography of the sample as well as foreign material that may be present, even when one might be talking about histological sections [28], [29].

Therefore, the diagnosis in PA could benefit with the use of electronic microscopy, scanning or transmission. However, scanning electronic microscopy is more used to give surface level information of the sample in analysis, and less to give cellular level information, or, even, intracellular information, like the transmission electronic microscopy does [30]. In PA, electronic microscopy techniques are rarely used when compared with light microscopy techniques.

There are several methodologies applied to the biological samples for the visualization of their internal structure in SEM: sample fracture, dissociation or microtomy [28]. Nevertheless, microtomy allows the information gathering using LM before SEM, because SEM allows the visualization of the sample surfaces and its topographic position in section [28].

Using microtomy techniques, it is possible to correlate with other types of microscopy, as well as the three-dimensional observation of the internal organization of the cell [28], [30]. With this method it is also possible to have a better orientation of the sample, as well as the position of the structures under study [28], [31]–[33].

The fact that using material prepared for the light microscopy can be used for ME and with good tissue preservation allows to obtain much useful information for the diagnosis by observation in ME of material included in paraffin [30], [34].

However, the samples for the SEM have to be treated so as to be vacuum stable and conductive, and for the TEM, in addition to these two conditions, they must also have the proper thickness. Vacuum stability and conductivity strongly condition the protocols of preparation of histological samples for EM.

The samples to be vacuum stable are prepared in the solid state [7]. That is, the water present in the biological samples has to be removed and replaced with a non-volatile medium in

vacuum. However, the removal of water leads to the redistribution of the components in the cell and the precipitation of others in the membrane or cytoskeleton, which can lead to misinterpretations [24], [29], [35].

One of the solutions is applying a solvent with superficial low tension: Hexamethyldisilazane (HMDS) [26], [36]. The sample dried with HMDS presents robustness and surface detail [37], [38]. In cells, the cytoplasmic areas are not affected and the nuclear area is easily distorted when the dehydration is done with alcohols. Given the less perinuclear stress and less damage at the nuclear level during sample drying, HMDS is more commonly used [26].

Other solution could be drying the biological samples by freeze-drying. The sample is treated with a liquid solvent (for example, T-butanol), which is then frozen and then sublimated [39]. This procedure avoids the existence of a liquid-air interface, which is the cause of surface tension. By sublimating the solvent, the surface tension is eliminated and there is no collapse of the most fragile structures [40]. The use of T-butanol instead of water does not allow the existence of artefacts such as cells damaged by water crystals [41], and it causes less tissue retraction when compared to the other dehydrating agents used [42].

The samples that are not conductive and that are to be visualized in the SEM present numerous artefacts, from the accumulation of the excess of charges [24], [43], [44]. To avoid these artefacts, the surfaces to be observed need to be coated with a conducting layer and grounded to mass of the microscope [24], [45]. In SEM, this is accomplished by coating the sample with a thin layer of metal or carbon, connecting to the mass with a conducting bridge [24], [29], [43], [46]–[48].

The metals used to cover the samples have good electrical conductivity. When they are under electron bombardment, they emit a large number of secondary electrons, which are then easily detectable by the detector [49].

Ionic liquids are salts that is in the liquid state at room temperature. Ionic liquids are being used in the investigation to visualize biological samples in the SEM, since the samples treated do not need to be dehydrated, therefore, not suffering any type of alteration to their *in vivo* state, besides of being stable in a vacuum environment [48], [50]. It is possible to visualize samples in the SEM with IL due that it its ionic conductivity, but not electrical conductivity. There are no issued of load effects in the sample [50], since it is the IL that is charged by irradiation of the electron beam in the vacuum chamber [48]. The images obtained with this procedure show a dark contrast when compared with the contrast of images obtained with metallic coating [48].

It is expected that the histologic routine procedures for LM can favour the correlation between images from either LM, SEM and TEM, because these images provide a complete morphologic detail of internal and external surfaces from biological samples, which allows the improvement of the diagnosis in PA [30], [51], [52].

II. Methods

A. Backscattered electron detector installation

The first task of the preent work was to install a backscattered electron detector in the SEM present in the laboratory, because the signal received form this detector gives information related to the relative chemical composition of samples. To install it in SEM, reverse-engineering was needed.

B. Experimental procedure for sample preparation

The second task of the preent work was the optimization of a protocol to visualize sample section on a slide in SEM. Thirteen sections for each sample in study (a paraffin-embedded pancreatic tissue, a paraffin-embedded cardiac tissue and a paraffin-embedded renal tissue, all from rat) were obtained using a Minot microtome.

In the optimization of the protocol, the three key-characteristics to visualize a biological sample in SEM were studied: the section thickness, drying and conductivity.

To analyse the thickness, four out of thirteen sections were used. One of them followed the procedure used in the laboratory to LM – 3μm of thickness and HE stain - and the rest, with different thickness - 5μm, 10μm and 15μm – followed the procedure used in the laboratory to SEM.

To analyse the drying, four out of thirteen section were used. One of them followed the procedure used in the laboratory to LM, and the rest, which had 10μm of thickness, it was tested the different drying methods: drying with low surface tension solvents (HMDS), drying with freeze-drying (T-butanol) and drying from water.

To analyse the conductivity, five out of thirteen section were used. One of them followed the procedure used in the laboratory to LM, and the rest, which had 10μm of thickness, it was tested different methods to increase conductivity: coating with IL without drying, drying with HMDS and coating with IL, drying with HMDS and coating with chromium (metal), and drying with HMDS and coating with chromium and IL.

Next, the optimized protocol was applied in six different rat tissues – heart, kidney, lung, liver, spleen and skeletal muscle. From each tissue two sections were obtained: one for LM – 3μm of thickness and HE stain – and one for SEM – optimized protocol - 10μm of thickness, drying with T-butanol and coating with chromium.

III. Results

The optimization of a protocol was needed because there is little literature referencing the use of this particular type of samples - histological sections visible under SEM. This optimization has three sample key-characteristics to study: thickness, drying and conductivity.

Every protocol (either optimization, either observation) had always a section for LM, because these sections allowed the identification of several structures of the samples.

The results were obtained using a secondary electron detector, as the installation of the backscattered electron detector in the SEM was not possible due to an unforeseen accident not related to this work.

A. Protocol optimization of sample preparation

1) Section thickness influence

For each sample, three sections with 5μm, 10μm and 15μm of thickness were analysed.

When analysing a 15μm thickness pancreatic section, its notion of tree-dimensionality is more visible, as expected (Fig. 1A, 1B and 1C). When observing a more tubular structure, we have to keep in mind that the detector is located in a lateral position. However, tilting the sample in the detector direction does not change the interpretation, neither the information that could be gathering from that (Fig 1D and 1H).

When a structure with more elements in which it is possible to ascertain its depth is analysed, like a blood vessel, the sections with 15μm yield more information, but less detailed when compared to 5μm or 10μm sections. In the 5μm section it is possible to observe that the section is superficial and that could be not enough to analyse the inside. However, in the 10μm section, that is already possible. When comparing both 10μm and 15μm section, it is visible that the structures around the blood vessel do not have a great definition, thus, losing information (Fig. 1E, 1F and 1G).

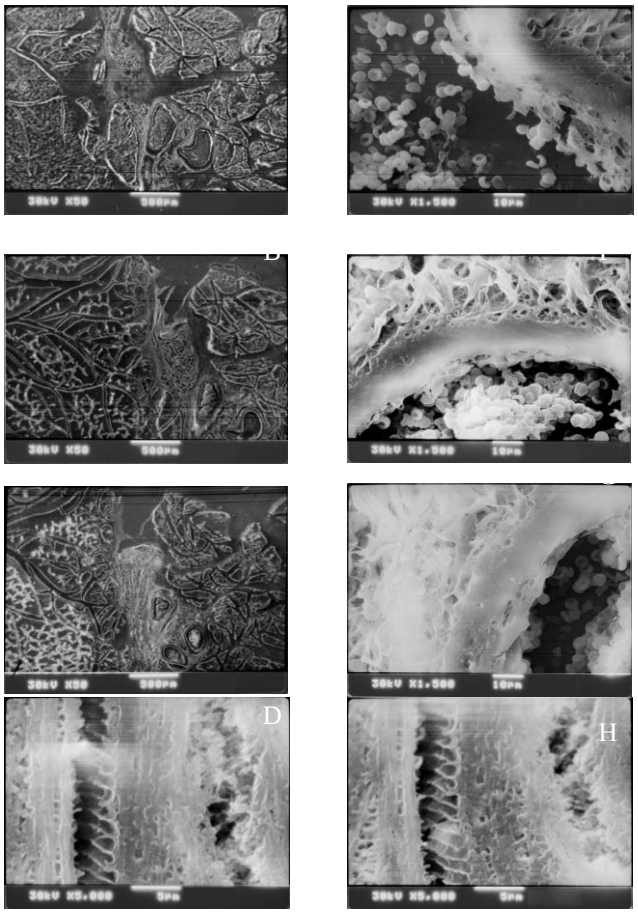

Fig. 1. A) 5μm section of pancreatic tissue; B) 10μm section of pancreatic tissue; C) 15μm section of pancreatic tissue; D) 5μm section of cardiac tissue without inclination; E) 5μm section of pancreatic tissue – blood vessel; F) 10μm section of pancreatic tissue – blood vessel; G) 15μm section of pancreatic tissue – blood vessel; H) 5μm section of cardiac tissue with inclination

2) Section drying influence

Three drying methods were analysed: HMDS, T-butanol and from water. Taking into account the results obtained previously, the sections have a thickness of 10μm.

At a lower magnification, it is possible to notice that with HMDS, the tissue appears to be more defined than the section with T-butanol. At a higher magnification, the areas with thinner structures are more present, or appear to be in greater number with the drying protocol with T-butanol (Fig. 2A, 2B and 2C).

The drying from water was used as a control group. Although it is possible to visualize structures at a lower magnification, when the magnification is increased, the small and more delicate structures do not endure the high water superficial tension and they collapse, leading to their not being so evident in the other sections.

When the blood vessels were analysed, at the same magnification, it is possible to observe that the section dried with HMDS appears to be more retracted (the tissue appears to be smaller) than the section dried with T-butanol, which is confirmed by the images observed at FESEM (Fig. 2D and 2E).

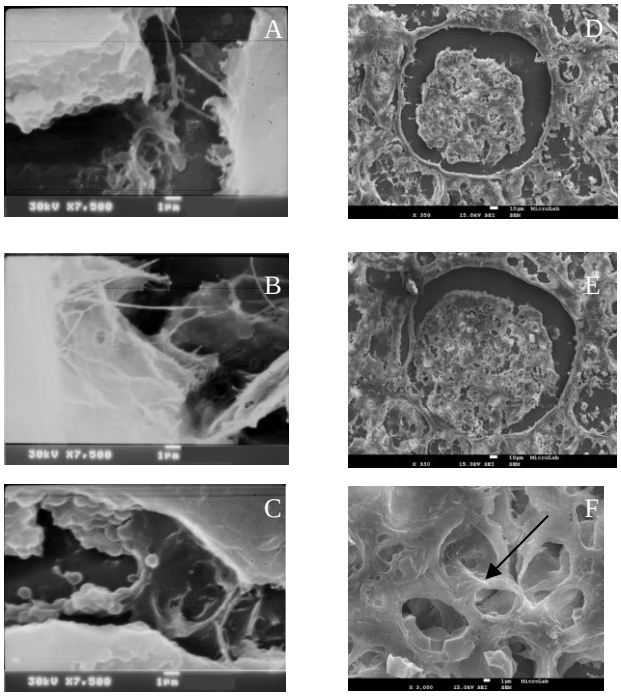

Fig. 2. A) Section drying with HMDS of pancreatic tissue – small strutures; B) Section drying with T-butanol of pancreatic tissue – small strutures; C) Section drying from water of pancreatic tissue – small strutures; D) Section drying with HMDS of renal tissue – glomerulus (FESEM); E) Section drying with T-butanol of renal tissue – glomerulus (FESEM); F) Section drying with T-butanol of renal tissue – (arrow) podocyte (FESEM).

3) Section Conductivity influence

Four methods to improve the section's conductivity were analysed: section coated with IL, with metal, with IL and metal, and another section coated with IL, but not dried. To analyse this characteristic, the sections are 10μm of thickness and dried with HMDS due to the ease of implementation of this protocol.

When the section was coated with IL but not dried, the contrast is different form when the section is dried. Due to that, in this case, it was no possible to visualise any type of structure and as such, it was determined that the section should be dried.

When the IL is applied, the contrast is lower than that with metal coating. The several structures or the fibre orientation are not visible, when compared with the metal coating (Fig. 3A, 3B and 3C).

At higher magnifications, the low contrast is still present, but that does not prevent a good visualization of the structures.

There is a higher intensity of the section coated with metal, than the section coated with both metal and IL and the section coated with only IL has a lower intensity (Fig. 3D, 3E and 3F).

When comparing the coating with metal with the coating with both methods, the contrast difference is almost inexistent, although the charges effects lines are present with the coating with both methods.

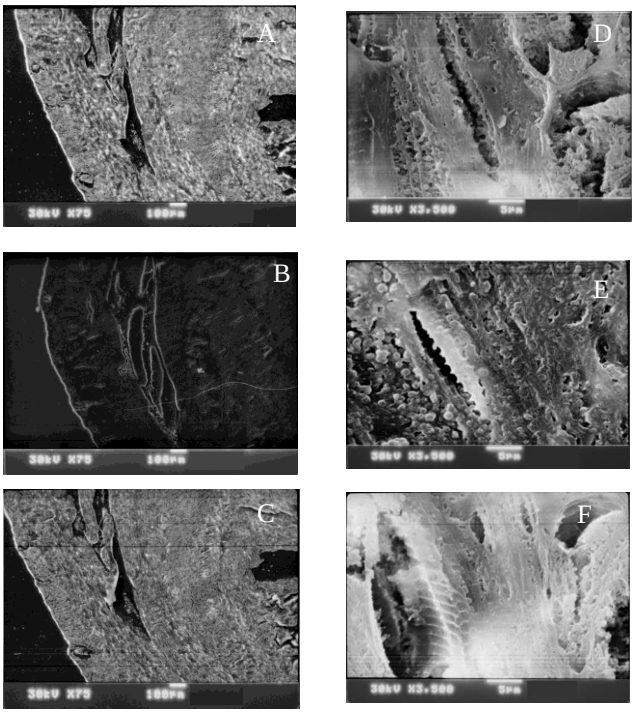

Fig. 3. A) Section coated with metal of cardiac tissue – low magnification; B) Section coated with IL of cardiac tissue – low magnification; C) Section coated with metal and IL of cardiac tissue – low magnification; D) Section coated with metal of cardiac tissue – high magnification; E) Section coated with IL of cardiac tissue – high magnification; F) Section coated with metal and IL of cardiac tissue – high magnification.

B. Observations after protocol optimization

After the optimization of the protocol, this protocol was applied to several types of rat tissue.

1) Heart

At a lower magnification, it is possible to notice the different muscular fibers orientation, and also the presence of blood vessels at the periphery of the section (epicardium). These blood vessels are easy to identify thanks to the presence of erythrocytes in their interior. It is also possible to visualize the blood vessel inner wall, especially when the sample is tilted in the detector direction (Fig. 4A and 4D).

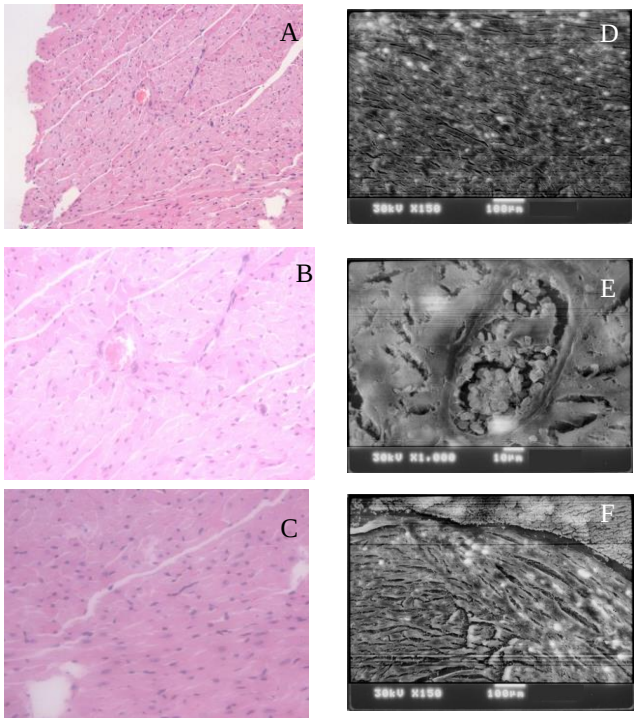

Fig. 4. A) Cardiac tissue (10X, LM); B) Blood vesel (20X, LM); C) Cardiac tissue – fiber orientation (20X, LM); D) Cardiac tissue (SEM); E) Blood vesel (SEM); F) Cardiac tissue – fiber orientation (SEM);

The presence of blood vessels, and, consequently, erythrocytes, gives an idea of dimension and also the preservation of the sample (Fig. 4B and 4E).

The heart is composed mainly by cardiac muscle (striated muscle), and that means that is easy to find the different fiber orientations – longitudinal when the fiber's length is observed and transversal when the fiber's interior is observed (Fig 4C and 4F).

2) Kidney

The kidney has a medullar part with more tubules and it has a cortical part with more glomeruli. The glomerulus is a ball of vessels enveloped by a capsule, where the visceral part has podocytes. (Fig 5A and 5B) Podocytes coat the blood vessels,

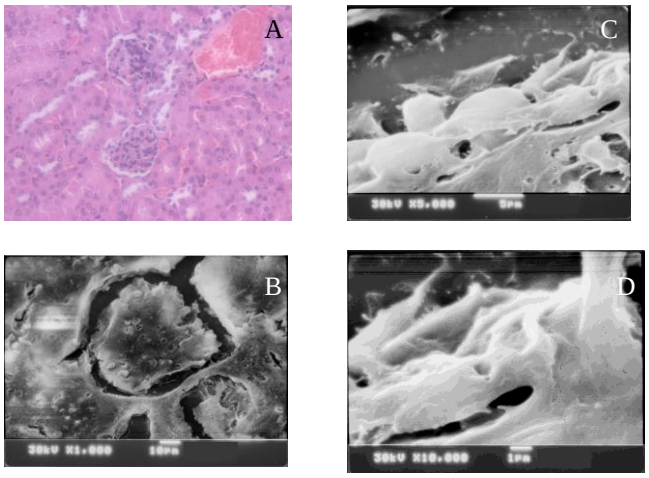

Fig. 5. A) Glomerulus (20X, LM); B) Glomerulus (SEM); C) Podocyte (SEM); D) Podocyte – detail pedicel (SEM).

and in section, it is possible to observe the pedicels (pedicular extension of podocytes) (Fig 5C and 5D).

3) Lung

The lungs, in their terminal portion, are composed by the pulmonary alveoli (Fig. 6A and 6C).

The identification of cartilage is easy in LM and SEM. At a higher magnification, it is possible to identify an amorphous material – cartilage (Fig. 6B and 6D).

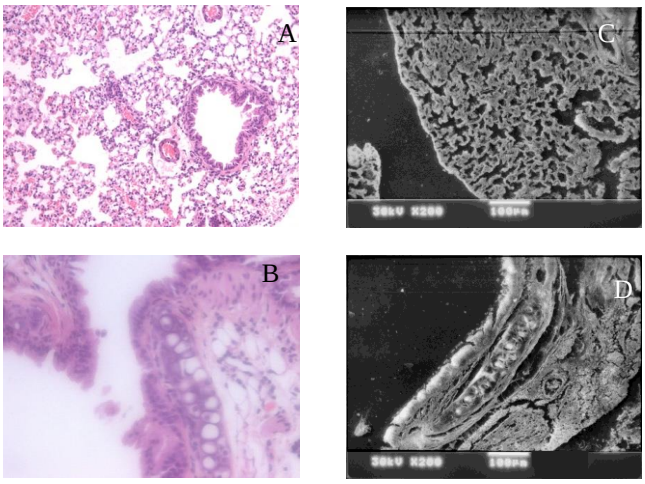

Fig. 6. A) Pulmonary alveoli (10X, LM); B) Cartilage tissue (20X, LM); C) Pulmonary alveoli (SEM); D) Cartilage tissue (SEM).

4) Liver

The liver is a massive organ, with (blood, lymphatic and hepatic) vessels, and also with hepatocytes. These hepatocytes are difficult to distinguish in SEM (Fig.7).

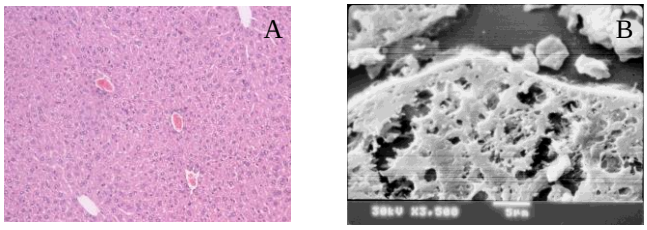

Fig. 7. A) Hepatic parenchyma (10X, LM); B) Hepatic parenchyma (SEM).

5) Spleen

The spleen is a tissue specialized in the germination of lymphoid cells. With the image from SEM it is not possible to distinguish the several germinative centers, like in LM.

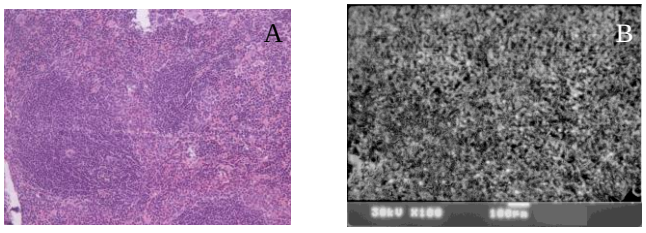

Fig. 5. A) Splenic parenchyma (10X, LM); B) Splenic parenchyma (SEM).

6) Striated muscle

The striated muscle, like the cardiac muscle, it is possible to observe the different fiber orientations.

IV. Discussion

A. Protocol optimization

From the few references found, it was necessary to optimize a protocol to visualize histological sections on SEM.

1) Section thickness

One of the characteristic that is important to have well defined is the section thickness, due to the three-dimensional appearance. In the pancreatic tissue analysed, it would have been interesting to discover the different types of hormonal tissue - endocrine (Langerhans islets) and exocrine tissue -, but that was not possible. The conclusion is that it is not easy to identify trough their superficial structure, but it is possible to identify by their content, like it is done in light microscopy with stains or in EM with high density markers detectable by backscattered electrons detector.

About the field depth, it was concluded that the section with 5µm of thickness is not ideal due to its lack of information, when compared with the other section thickness in study. The same happens with surrounding structures, which cannot be perceptible if the structure has bottom due to its cut.

Comparing the sections with 10µm and 15µm, there is not great difference, being the only exception the section with 15µm of thickens that could have given more information, but that did not occur.

At the cardiac tissue, three layers should be seen: the epicardium which has squamous epithelium, blood vessels, adipose and connective tissue; myocardium, which has cardiac cells, connective tissue and intercalated discs; endocardium, which has adipose and connective tissue. During the processing of the section to LM visualization, the adipose tissue was not preserved, and so it was not visible in SEM with this protocol. However, it was possible to distinguish the epicardium tissue due to the presence of blood vessels.

Regarding the myocardium, there will be different layers of muscle tissue with different orientations. At a higher magnification, it is possible to see some extensions, which are the intercalary discs.

In conclusion, the section with 10µm thickness was chosen because of the better interpretation of the information gathered, as well as the technical difficulties in the preparation of samples in order to obtain sections with 15µm in thickness with the best quality possible, as seen in the references [30] and [33] (Table I).

2) Section drying

TABLE I

COMPARATIVE TABLE OF THE DIFFERENTE SECTION THCINKENSS

	5µm	10µm	15µm
Struture identification	Weak	Strong	Moderate
Field depht	Moderate	Strong	Weak

In the protocols for the visualization of biological samples in SEM, the samples need to be dry. A possible solution is freeze-drying with T-butanol, which can be performed with equipment commonly available in laboratories, and it has, therefore, been adopted. Drying the section with HMDS was also used due to its easiness and widespread adoption. The

protocol with drying from water served as a control, in order to compare with the results from the use of this protocol.

While analysing the structural collapse of the samples, it was visible that this is present in the method that uses drying from water, as expected, and less present in the HMDS method. The T-butanol method has the best results in this characteristic. Still, it is possible to see a few characteristics of the tissue when drying the section from water, as long as these do not contain small or fragile structures.

Regarding tissue retraction, it is possible to study this tissue alteration when analysing the same field of the sample. For example, analysing the glomeruli at the same magnification, the glomerulus of the section dried with HMDS is slightly smaller than the one from the section dried with T-butanol. This artefact may be due to the effects of the surface tension in the sample being air-dried, having a liquid-air interface. With T-butanol, it is sublimated, there being no interface, not altering the preservation of the sample, as described in the reference [42].

Analysing the structural collapse of the samples, it was noted that this was present in the method that uses drying from water, as expected, and less present in the HMDS method. The method with T-butanol, is the one that causes the least collapse, as described in the references [37] and [53]

In general, when low magnifications were used, it was not possible to distinguish the best protocol. However, with higher magnifications, to see smaller and fragile structures, these structures are better preserved with T-butanol.

One can conclude that weighing the required execution time, the drying with HMDS is good, taking into account the execution time that is required, when compared to the T-butanol method. However, if it is desired to visualize very thin, small and fragile structures at high magnifications, the best method is the latter, because it was the one that presented better results when the sections were seen in a FESEM (higher resolution microscope).

In summary, drying sections with T-butanol are ideal for the visualization of LM-prepared biological tissue in the SEM, especially when it is necessary to visualize small and fragile structures in the condition tested (Table II).

TABLE II

COMPARATIVE TABLE OF THE DIFFERENT SECTION DRYING METHOD

	Water	HMDS	T-butanol
Structural collapse	Strong	Moderate	Weak
Structure definitiont	Weak	Moderate	Strong
Tissue retraction	Strong	Strong	Weak

3) Section conductivity

For the visualization of biological samples in SEM, they must have a conductive surface. To do that, the sample is coated with a conductive layer, generally metallic. However, IL is often used for biological samples, because they are miscible in water and the sample became conductive. It was decided to test the method to see if in this case, with histological sections this would work. Should it have work, it would not be necessary to use the drying step of the sample [48].

The methodology of using IL without first drying the biological sample should not be applied, as it was not possible to visualize any structure or tissue when placed in the SEM. One of the factors that justify this protocol not working may be the need to rehydrate the section, because IL needs water, which a paraffin section does not have.

When the section is covered with IL, it does not present great detail and it presents a different contrast, when compared with the other protocols. This different contrast is described in the references, since IL is not an electric conductor, but rather ionic, and may also present little signal intensity, which interferes with the interpretation of the image due to the noise produced.

When the section is covered with metal (chromium, in this case) the charges effects are visible, but this problem is overcome when a denser coating is applied to the sample.

When combining the methods, it is possible to observe the reduction of the charges effects. It is possible to observe more contrast and more charges effects, which is not beneficial for good image quality.

In conclusion, the metal-coated (chromium) sections are the best under these conditions (Table III).

TABLE III

COMPARATIVE TABLE OF THE DIFFERENT SECTION CONDUCTIVITY

	IL	Metal	IL and Metal
Signal intensity	Weak	Strong	Strong
Charges effects	Weak	Moderate	Moderate

B. Observations after protocol optimization

After optimization of the protocol for the visualization of histological sections in SEM, the same protocol was applied in several tissues, to verify its practicability in different tissues.

Some tissues have more structures than others, making it easier to analyse them

1) Heart

The heart is a tissue with several structures visible in the SEM. The different orientations of the fibers, the connections between cells, the interior of the vessels, are almost all structures that are not very visible in light microscopy but visible in EM. When they are visible in the SEM, it will be possible to identify cellular changes that are not visible in light microscopy.

Comparing the structures seen at a low magnification in both microscopes, it is possible to notice the capacity that the SEM presents to have a resolution that goes from that presented by LM. This allows the microscopist to have a facilitated orientation of the tissue and from there to analyse structures at a much higher magnification.

2) Kidney

The kidney is a tissue with several structures visible in SEM. The easiness of magnification in the glomerulus allows the visualization of cells like the podocytes, as well as the pedicels. This make the use of the SEM together with the LM useful to study the structure of the glomerular-capillary membrane.

The visualization of sections with higher resolution, such as the images obtained in the SEM, facilitates the identification of certain structures, that provide information about physiology and renal pathologies.

The study of podocytes and their pedicels, which is something that is done usually in TEM, can be done in SEM, and there are pathologies that are related to changes in these cells regarding TEM [54]; the SEM allows a simpler and faster processing, leading to a diagnosis in less time.

3) Lung

The lung is also one of the tissues analysed in which is observed several structures that can give more information. However, with the signal of secondary electrons only, the obtained information is not different from the one obtained in LM, as it is shown when comparing the two microscopies: the immediate identification of the pulmonary alveoli, the only difference being the depth perception present in the SEM, and the identification of the cartilage material –in LM by the different affinity of the stain and in SEM by the different interaction with the electrons, becoming brighter.

The use of other signals, such as backscattered electrons or X-rays, offers the possibility of identifying and analysing particles in the lung parenchyma [55].

4) Liver

The liver, when viewed in the SEM, under the experimental conditions, does not provide more information than that obtained in LM. In fact, the use of other stains for the LM gives more information than in the SEM, as the example of silver stains for reticulin [5]. It should be noted that the SEM can also be used, for example with backdrops electron images to analyse sections stained with heavy metals, such as silver.

5) Spleen

The spleen, being a lymphoid germinative tissue, is composed of a variety of different cells. However, these are not easily identified in SEM, because on the surface, they do not differ between them. Thus, as in the liver, the information that can be obtained in the SEM is not an added value in the conditions of the experience, when compared with the information obtained by the other microscopies.

6) Striated muscle

In skeletal muscle, it is possible to observe the different orientations of the fibers. But as with the liver and spleen, there are no more structures to see in the muscle that can give more information about cell changes. The striated muscle is not a very common tissue in PA, and a biopsy of this tissue is usually made only to determine some neurological pathology.

V. Conclusions

According to this results, it is possible to conclude that the use of SEM in the diagnostic practice using correlative microscopy is practicable. However, some tissues did not show benefits with the methodology used for normal tissue observation. The study of pathological specimens, particularly those produced by foreign bodies, may eventually reveal applications with indications for the use of SEM.

The few references existing regarding the methodology that could be applied in this type of specimens (paraffin-embedded tissue) made it necessary to adapt the existing protocols for this type of material - in particular in the observation of histological sections.

Obtaining histological sections so that can be seen in the SEM showed to be a technique that has value in the diagnosis. The visualization of certain structures in greater magnification, but still, without any disruption of the sample seen in LM, can provide vital information for a better diagnosis.

The SEM available in the laboratory had only installed the secondary electron detector, which was the one used, and from which all these conclusions were withdrawn. However, the signals that could be obtained by the backscattered electron detector or even the X-ray energy dispersion spectroscopy would provide another type of information, also possibly relevant for the diagnosis of various pathologies. Nevertheless, several aspects of pathologies would have to be studied to better classify them using these detectors.

Taking into account the conclusions it is considered pertinent to make some suggestions for future studies: study other characteristics of the sample, in particular sections stained with heavy metals with the SEM, to explore other potentialities; study other types of tissue, as well as to analyse tissues already with pathologies, to realize the true impact of this technique, in the diagnosis of the same ones; study the different types of signals that can be obtained from an SEM besides the secondary electrons - backscattered electrons, X-ray energy dispersion spectroscopy, and others.

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