UNIVERSITY POLITEHNICA OF BUCHAREST
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Physico-chemical study of tissue samples and the impact of pre-analytical variables in Pathological Anatomy

ABSTRACT DOCTORAL THESIS

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Content

Keywords: pre-analytical variables in pathology, tissue processing protocols, dehydration, fixation, clarification, residual water, tissue artifacts, biopsy pads, section retention, tissue microarrays, cost analysis.

Note: The notations of the chapters, sub-chapters and respectively of the figures, tables and graphs in this document are the same as those in the doctoral thesis.

INTRODUCTION

Quality monitoring in the histopathology department is classified into three phases, pre-analytical, analytical and post-analytical, to cover different stages of the entire testing cycle. Literature review of histopathology quality assessment studies revealed that previous reports have focused mainly on analytical aspects, with limited studies on pre-analytical phase assessment. The pre-analytical phase comprises several stages of sample processing and handling, thus allowing for a sufficient amount of error. Due to its critical nature and limited studies in the past to assess quality in the pre-analytical phase, it deserves further attention.

The aim of this study was to analyze the influence of different pre-analytical variables (fixation protocol, processing protocol, solutions used in the processing protocol, tissue type) on the quality of tissue samples analyzed the Pathological Anatomy laboratory.

In order to fulfill our proposed goal, we developed and executed a series of experiments in which we tested several factors that can influence the quality of the final histological preparation (the microscope slide on which histologically stained sections are displayed, ready to be interpreted by the specialist). In the first part of the experiments, we tested the influence of four processing protocols on biopsies of different rabbit tissues (two protocols frequently used in histology laboratories that use xylene as a clearing agent and two protocols developed in the Themis Pathology laboratory that use *n*-butanol as the clarifying agent). The second experiment consisted in the study of the impact of histological sponges (used in laboratories to maintain the integrity and flatness of tissue biopsies and to eliminate the risk of losing them during processing) on the degradation of the dehydration solutions and implicitly on the fitness of the resulting microscopic preparations for providing an accurate diagnostic.

The experiments continued with studies that looked at the influence of the fixation protocol, the processing protocol and the influence of the type of tissue processed on the accidental detachment of the sections from the microscope slide during immunohistochemical staining, due to the harsh protocols used for this type of assay. The last part of the experiments focused on the implementation of a customized method of multiplexing tissue samples in the histology laboratory (tissue matrix/tissue microarray) and the cost analysis once this technique is introduced in the laboratory.

All these studies helped us to develop an optimal protocol for processing tissue biopsies that was further implemented in two preclinical studies.

The thesis is structured in three parts and includes 9 chapters. The bibliographic part (4 chapters), the experimental part – original contributions (4 chapters), and the application part (preclinical studies) (1 chapter). The first part presents a literature study regarding the research topic, the second part represents the original experimental part, and the third part presents the results of two preclinical studies following the implementation of the optimal tissue sample processing protocol developed at the experimental part.

The first part represents the literature study which is further structured in four chapters and presents the literature data regarding state of the art in the field of pre-analytical histology. In **chapter 1** (PRE-ANALYTICAL VARIABLES IN PATHOLOGICAL ANATOMY) the specialized literature study is presented describing different pre-analytical factors that are associated with the histological protocol (dehydration, clarification, impregnation and incorporation in a support medium), the processing methods, as well as the

effect of these factors on the final results. **Chapter 2** entitled HISTOLOGICAL SPONGES includes some generalities regarding the influence of this less studied pre-analytical factor on tissue samples. **Chapter 3** (PRE-ANALYTICAL VARIABLES THAT CAN INFLUENCE THE RETENTION OF SECTIONS ON THE MICROSCOPE SLIDE DURING IMMUNOHISTOCHEMICAL STAINING) presents the scientific literature examining the factors that influence the retention of tissue sections on the glass slide during immunohistochemical staining. In **chapter 4** (TISSUE MICROARRAY - TISSUE MATRICES) there are reviewed different methods of multiplexing paraffin blocks (collecting several biopsies from "donor" blocks to a single "recipient" block). This method gained popularity in the last 20-30 years in research laboratories but only recently was considered for clinical use. In this chapter there are presented the principles of obtaining tissue matrices, the advantages and the potential clinical applications.

The second part of the thesis (chapters 5-8) presents the experimental part and original contributions. In each chapter, different pre-analytical factors and their influence in histology are studied. **Chapter 5** (DETERMINATION OF RESIDUAL WATER IN PROCESSING SOLUTIONS AND PROCESSED TISSUE SAMPLES) examines a number of research studies examining the influence of four processing protocols (two protocols commonly used in histology laboratories that use xylene as a clarifying agent and two protocols developed within the Themis Pathology laboratory, which use *n*-butanol as a clearing agent) on rabbit tissue biopsies (liver, kidney, spleen, jejunum, skeletal muscle, testis, ear). The impact of the processing protocols was evaluated by quantifying the amount of water extracted from the tissue samples by the dehydration solutions (Karl Fisher volumetric titration) as well as the amount of residual water remaining in the biopsies (thermogravimetry on the processed tissue exposed to moisture-drying cycles). The quality of the processing protocols was also evaluated by careful examination of the quality of the paraffin blocks and of the obtained histological stainings. The results showed us that the protocols using *n*-butanol as a clarifying agent gave the best results in terms of minimal residual water and superior morphological and immunohistochemical stainings. Chapter 5 is further divided into sub-chapters presenting the materials and methods used, the results obtained from the determinations and the conclusions of the study. **Chapter 6** (INFLUENCE OF HISTOLOGICAL SPONGES IN PROCESSING PROTOCOLS OF TISSUE SAMPLES) presents the study on the influence of histological sponges (of various porosities and grades of reticulation) on the contamination/degradation of the solutions used in biopsy processing protocols as well ast their effects on the quality of topographic and immunohistochemical staining of the resuling slides. This chapter is structured in subchapters: materials and methods, results and conclusions. **Chapter 7** (THE INFLUENCE OF PRE-ANALYTICAL VARIABLES IN THE RETENTION OF SECTIONS ON THE MICROSCOPE SLIDE DURING IMMUNOHISTOCHEMICAL STAININGS) is covering the pre-analytical factors that can determine the detachment of the sections from the microscope slide during immunohistochemical staining, due to the harsh treatments used for antigen retrieval. The tested parameters are fixation, tissue processing protocols, biopsy size and tissue type. The main findings are that inappropriate processing protocols result in the sections detaching from the glass slide as follows: 29% (5 mm biopsies), 24% (3 mm biopsies) and 48% (1 mm biopsies), while biopsies with delayed fixation (biopsies left for 12h at room temperature prrios to room temperature fixation for 252h with 10% NBF) are detached from the glass slide up to 41% (1 mm biopsies, particularly those harvested from

stomach and tongue). This chapter is further divided into sub-chapters: materials and methods, results and conclusions. **Chapter 8** entitled COST ANALYSIS IN THE HISTOLOGY LABORATORY: TMA VS CLASSICAL METHOD presents the cost analysis in the histology laboratory if tissue arrays (TMA) are used instead of the classical method. Depending on the model used, the total cost of histology services can be reduced by up to 91%. Chapter 8 is divided into the following subchapters: materials and methods, results and conclusions.

The third part - APPLICATIONS, consists of **Chapter 9** and presents the results obtained following the implementation in two preclinical studies of the tissue processing protocol that provided the best results in the experimental part of this work (the protocol that used *n*-butanol as a clarification solution). In the first study, three anti-inflammatory drugs were tested in an experimental model of psoriasis, and in study 2, the immunological effect of products from the Imunoinstant range (Hyperimmune Egg, Imunoinstant G, Imunoinstant multiple) was tested. The processing protocol used allows to obtain paraffin blocks and microscope slides of very good quality, facilitating an easy and accurate measurement of the granular layer, a measurement necessary to determine the degree of orthokeratosis in study 1, and an accurate histological evaluation of the stained sections with hematoxylin-eosin in the case of study 2.

At the end of the thesis, the original contributions and the general conclusions of the doctoral thesis are presented. The thesis ends with a Bibliography chapter containing 120 bibliographic references and a list of original scientific publications.

THE OBJECTIVES OF THE THESIS

The general objective of this doctoral thesis was the physicochemical study of tissue samples and the impact of pre-analytical variables in Pathological Anatomy.
The achievement of this objective was realized in the following stages:
1. Determining the water content of the tissue sample at different stages of processing, by equilibrating the samples with universal solvents and determining the amount of water in the solvent used;
2. Determination of the fluctuating water content in the paraffinized tissues by exposing them to moisture/drying cycles and weighing the samples with a high-precision analytical balance;
3. Determination of the water content of paraffinized tissues by thermogravimetry;
4. Quantification of water extraction from the tissue during different stages of processing;
5. Evaluation of the influence of the histological sponges on the processing solutions and implicitly on the tissue samples;
6. Analysis of the influence of different pre-analytical parameters (tissue type and biopsy size, fixation and processing protocols, antigen retrieval solutions) on the accidental detachment of sections from the microscope slide during immunohistochemical staining;
7. Generation of customized tissue microarrays from paraffin-embedded samples;
8. Use of sectionable tissue microarrays of tissue samples in the histology laboratory and the cost analysis versus conventional methods.

PART TWO – EXPERIMENTAL STUDY

CHAPTER 5. DETERMINATION OF RESIDUAL WATER IN PROCESSING SOLUTIONS AND IN PROCESSED TISSUE SAMPLES

The main factor responsible for the appearance of artifacts following the processes of dehydration, clarification and paraffin infiltration/embedding of biological preparations for histological analysis is the residual water left in the sample and/or exposure to atmospheric oxygen. All studies to date confirm that, regardless of the processing protocol used, minutes amounts of residual water remain in the paraffinized tissue samples. Therefore, the aim of any tissue processing protocol is to extract as much water as possible from the sample, regardless of the solvents or methods used.

The aim of the present study is to find an optimal dehydration protocol that does not negatively affect the chemistry, morphology, antigenicity, architecture, RNA and DNA of the tissue.

In our research laboratory (Themis Pathology, Voluntari, Ilfov) we developed a three-day long processing protocol, at room temperature, which uses *n*-butanol as a dehydrating/clarifying agent. Butanol is a universal solvent partially miscible with water and a good intermediate between alcohol and paraffin. To accelerate the diffusion of solvents in the tissue (and shorten to total duration of tissue processing), we tested a second protocol with *n*-butanol, performed at 37 °C, for only 10 hours and 30 minutes. Alternative protocols are two conventional xylene protocols, performed at room temperature and at 37 °C, respectively.

The testing of the four processing protocols was carried out on biopsies of different types of New Zealand rabbit tissue, and their choice was made according to the composition and structure of the tissue. For biopsies, we chose organs with different water content (high water content - testicle, low water content - ear, jejunum), homogeneous organs (spleen, liver, muscles) but also heterogeneous (kidneys). Depending on the structure of the tissue, the diffusion rate of the solvents is different, so the tissues chosen for testing were tissues with fast diffusion (kidney, jejunum) and slow diffusion (testis, liver, ear, muscle, spleen) [54, 58].

In order to evaluate the quality of the histological preparations obtained following the four processing protocols, as well as the influence of the morphology and initial composition of the tissue on the performance of the four protocols, several determinations were made, so that chapter 5 is structured as follows:

- Determination of the water content of the dehydration / clarification solutions using four different histological processing protocols (by Karl Fischer volumetric titration);
- Determination of the fluctuating water content in the paraffinized tissue (by exposing it to humidity/drying cycles) by weighing with a high-precision analytical balance;
- Determination of the water content of paraffinized tissues by thermogravimetry;
- Assessment of water content within the tissue sample at different stages of processing (after equilibrating the samples with universal solvents, the water in the used solvent is determined by Karl Fischer volumetric or coulometric titration);
- Quantification of water extraction from the tissue in different stages of processing.

For the subsequent analysis (topographical and immunohistochemical staining) of the tissue samples processed with four processing protocols, we used the multiplexing method (less expensive and faster). This consists of using sectionable matrices that incorporate

multiple biopsies into a single paraffin block. This stage was carried out after obtaining the "donor" blocks (196 donor blocks).

The role of the processing protocol can be seen in the determinations of carry-over water in the processing solutions (Table 5.3).

During the dehydration stage, ethyl alcohol has the role of extracting water from the tissue. It can be seen that up to step 5 [ethanol 100 % (1)] approximately the same amount of water is extracted from the tissue for all four protocols. For protocols I and II the dehydration is continued with another exchange of 100 % ethanol, and for protocols III and IV the dehydration is continued with the first exchange of *n*-butanol (step 6), since this alcohol is miscible with water.

The amount of water extracted from the tissue by the two solutions is different: absolute ethyl alcohol is dehydrating the tissues more efficiently/quickly than *n*-butanol.

On the other hand, after the last exchange of absolute alcohol (Protocol I and Protocol II), the small amount of water remaining in the tissue will no longer be extracted during the clarification stage, xylene being practically immiscible with water.

For Protocol III and Protocol IV, dehydration will continue during the clarification step because the *n*-butanol will continue to slowly extract the remaining water in the tissue.

After the last clarification step, a higher concentration of residual water in the clarification agent in the protocols using *n*-butanol was observed.

It is well known that universal solvents (butanol, dioxane, tetrahydrofuran, etc.) facilitate better histological preparations, particularly in the case of fragile tissues like (brain, cephalopods, plants (because the dehydration is slower, delicate, and at the same time complete) [23].

Table 5.3. The concentration of water in the processing solutions used during the four protocols tested. The amount of water extracted was determined usingthe Karl Fischer coulometric method. Steps from 1 to 8 represent the processing solutions corresponding to the protocols described in section 5.1.3. The solutions from steps 1-5 represent the dehydration solutions, and the solutions from steps 7-8 represent the clarification solutions.

Processing stage	Water concentration in the processing solutions			
	Protocol I	Protocol II	Protocol III	Protocol IV
1	35.37	34.17	34.61	35.91
2	21.64	22.67	21.85	12.44
3	7.576	5.717	6.561	6.466
4	6.561	6.775	6.346	-
5	0.403	0.392	0.399	0.579
6	0.156	0.148	0.152	-
7	0.031	0.035	0.149	0.326
8	0.029	0.012	0.155	0.137

5.2.2 Determination of the amount of fluctuating water in paraffin tissue samples

The amount of fluctuating water in the tissues processed with the four protocols tested was initially determined by exposing the samples to cycles of high humidity and cycles of low humidity (drying). To obtain the saturation value, the samples were allowed to equilibrate in the climatic enclosure for 4 days for each cycle. The difference between the values obtained

when weighing at high humidity and weighing at low humidity (drying) represents the fluctuating water in the tissue.

The results (Table 5.4) showed statistical significant differences between the four protocols depend as well as between the various types of tissue examined. While the amount of fluctuating water is fairly large for liver and muscle ($p < 0.05$) for intestine, kidney, ear and testis ($p > 0.05$) the values are much smaller.

On average, the amount of water that is absorbed and then removed from the tissue during the wet/dry cycles, regardless of the type of organ, is lower in organs processed with protocols III and IV.

Table 5.4. The amount of fluctuating water (in grams) in processed tissue (liver, intestine, muscle, kidney, ear, testis). Values represent the mean of seven determinations. SEM represents the standard error of the mean. Differences were considered significant if $p < 0.05$ (ANOVA test)

Organ		Grams of water fluctuating in the tissue				ANOVA test
		Protocol I	Protocol II	Protocol III	Protocol IV	p value
Liver	Mean	0.025	0.038	0.025	0.021	$p < 0.05$
	SEM	0.002	0.003	0.002	0.002	
Intestine	Mean	0.010	0.011	0.011	0.010	$p > 0.05$
	SEM	0.001	0.002	0.002	0.001	
Muscle	Mean	0.015	0.016	0.015	0.010	$p < 0.05$
	SEM	0.001	0.001	0.002	0.002	
Kidney	Mean	0.013	0.017	0.013	0.013	$p > 0.05$
	SEM	0.001	0.001	0.001	0.001	
Ear	Mean	0.025	0.026	0.026	0.024	$p > 0.05$
	SEM	0.003	0.002	0.005	0.003	
Testis	Mean	0.014	0.038	0.015	0.023	$p > 0.05$
	SEM	0.002	0.005	0.002	0.004	

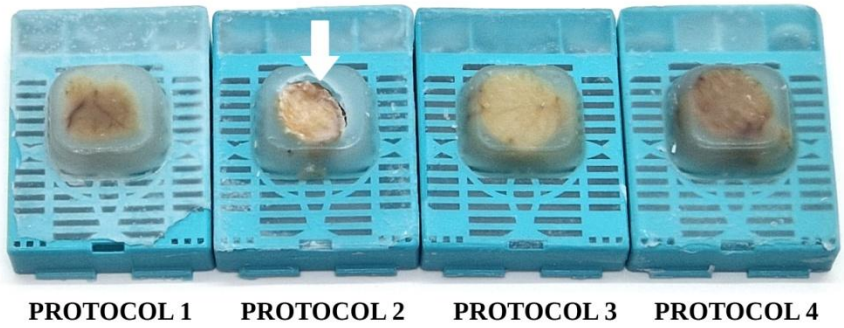

Figure 5.8. Paraffin blocks containing testicular biopsies: Protocol 1–Protocol 4. White arrow indicates a depression of tissue samples in the paraffin block, clear sign of excessive residual water/insufficient tissue processing

The results obtained from the exposure of the tissue samples to moisture/drying cycles were correlated with the results from the visual examination of the paraffin blocks. A few days after making the paraffin blocks, we noticed that some of the blocks containing the testicular biopsies had a depression on their surface, indicative of insufficient paraffin

infiltration within tissue samples caused by incomplete/defective processing. This was observed only in tissues processed with Protocol 2 (Figure 5.8). Incomplete infiltration followed by shrinkage of the tissue samples was more evident among testis samples, but was also occasionally observed for the other types of organs: less marked for ear and skeletal muscle, while the other organs (liver, kidney, spleen, jejunum) seemed to be better processed.

5.2.3. Determination by thermogravimetry of the amount of water in the tissue samples

After determination of the fluctuating water in the tissue samples (exposing them to cycles of humidity/drying and weighing), a total of 14 testis samples were used for a secondary determination by thermogravimetric analysis (TGA).

The thermogravimetric results (Table 5.5) confirmed that the samples that used *n*-butanol as a clarifying agent contain the smallest amounts of residual water: 0.011 ± 0.001% vs 0.04 ± 0.004% when compared with the values obtained for the tissue samples clarified with xylene ($p < 0.05$, the values are significant).

Table 5.5. Thermogravimetry results for testis samples processed with xylene and *n*-butanol. The results are expressed in percentages and represent the mass losses in the temperature range 100–120 °C and the inorganic residues after the temperature of 700 °C

Sample	% mass loss		% inorganic residue	
	xilen	*n*-butanol	xilen	*n*-butanol
1	0.02	0.02	5.5	3.4
2	0.05	0.01	4.7	2.9
3	0.03	0.01	7.5	3.2
4	0.05	0.01	1.7	5.6
5	0.05	0.01	1.3	4.8
6	0.04	0.01	2.6	1.2
7	0.04	0.01	4.5	4.4
Average	0.04	0.011	3.97	3.64
SEM	0.004	0.001	0.84	0.55

5.2.4 Statistical analysis of the water extraction from the tissue in different stages of processing

The results of the ANOVA test are specified in Table 5.6, where $X_{m0} = X_{m,FF}$ and $X_{m,fin}$ are the initial and final amounts of the water mass ratio (X), and W (%) represents the percentage of extracted water defined by equation (5.1).

$$W = 100\frac{\left(X_{m0} - X_{m,fin}\right)}{X_{m0}} \tag{5.1}$$

The results summarized in Table 1 highlight that, for all tissues, processing protocol 4 is the most effective:

(i) for liver, $X_{m,fin,4} = X_{m,butanol(2)} = 0.04$, significantly lower (3-7 times) than the $X_{m,fin,i}$ (i=1,2,3) corresponding to the other 3 processing protocols (0.12-0.26), whereas $W_4 = 99.0\%$ was slightly higher (1-5%) than $W_i = 93.9\text{-}98.3\%$;

(ii) for intestine, $X_{m,fin,4} = X_{m,butanol(2)} = 0.19$ was significantly lower (about 3 times) than the $X_{m,fin,i} = 0.47\text{-}0.49$ and $W_4 = 98.0\%$ was higher (3-10%) than $W_i = 89.1\text{-}95.2\%$;

(iii) for skeletal muscle, $X_{m,fin,4} = X_{m,butanol(2)} = 0.09$ was significantly lower (3-5 times) than the $X_{m,fin,i} = 0.23\text{-}0.45$ and $W_4 = 98.4\%$ was higher (3-6%) than $W_i = 92.6.1\text{-}96.0\%$;

(iv) for kidney, $X_{m,fin,4} = X_{m,butanol(2)} = 0.11$ was significantly lower (2-5 times) than the $X_{m,fin,i} = 0.26\text{-}0.54$ and $W_4 = 98.3\%$ was higher (2-7%) than $W_i = 91.5\text{-}96.6\%$;

(v) for the ear, $X_{m,fin,4} = X_{m,butanol(2)} = 0.08$ was significantly lower (3-12 times) than the $X_{m,fin,i} = 0.23\text{-}0.93$ and $W_4 = 97.9\%$ was higher (4-25%) than $W_i = 78.5\text{-}94.5\%$.

Moreover, for processing protocol 1, the extraction with 100% ethyl alcohol solution could be omitted, because there were no statistically significant differences among $X_{m,A95(2)}$, $X_{m,A100(1)}$, and $X_{m,A100(2)}$. Similarly, for processing protocol 2, the extraction with 95% ethyl alcohol solution could be performed in one step, because there were no statistically significant differences between $X_{m,A95(1)}$ and $X_{m,A95(2)}$.

Table 5.6. Initial ($X_{m0} = X_{m,FF}$) and final ($X_{m,fin}$) means of water mass ratio (X) in different tissues and processing protocols, and comparisons among the means of X (X_m) after water extraction with different solvent types/concentrations and extraction step numbers [(1), (2), and (3)]

Tissue	Processing protocol	X_{m0}	$X_{m,fin}$	W (%)	Observations
Liver	1	4.05	0.12	97.0	There are no statistically significant differences among $X_{m,A95(2)}$, $X_{m,A100(1)}$, $X_{m,A100(2)}$ and $X_{m,xylene(1)}$
	2	4.28	0.26	93.9	There are no statistically significant differences between $X_{m,A95(1)}$ and $X_{m,A95(2)}$, and $X_{m,A100(2)}$ and $X_{m,xylene(1)}$
	3	4.96	0.12	98.3	There are no statistically significant differences between $X_{m,A100(1)}$ and $X_{m,butanol(2)}$, and $X_{butanol(2)}$ and $X_{butanol(3)}$
	4	3.88	0.04	99.0	All X_m corresponding to extraction with different solvent types/concentrations and extraction step numbers are significantly different
Intestine	1	9.89	0.47	95.2	There are no statistically significant differences between $X_{m,A100(1)}$ and $X_{m,xylene(2)}$
	2	9.84	0.48	95.1	$X_{m,A100(1)}$ and $X_{m,xylene(2)}$ are significantly different
	3	4.48	0.49	89.1	There are no statistically significant differences between $X_{m,A100(1)}$ and $X_{m,butanol(3)}$
	4	9.43	0.19	98.0	$X_{m,A100(1)}$ and $X_{m,butanol(2)}$ are significantly different
Muscle	1	5.7	0.23	96.0	$X_{m,A100(1)}$ and $X_{m,xylene(2)}$ are significantly different
	2	6.09	0.45	92.6	$X_{m,A100(1)}$ and $X_{m,xylene(2)}$ are significantly different
	3	6.1	0.26	95.7	$X_{m,A100(1)}$ and $X_{m,butanol(3)}$ are significantly different
	4	5.59	0.09	98.4	$X_{m,A100(1)}$ and $X_{m,butanol(2)}$ are significantly different
Kidney	1	7.08	0.4	94.4	There are no statistically significant differences between $X_{m,A100(1)}$ and $X_{m,xylene(2)}$
	2	6.37	0.54	91.5	$X_{m,A100(1)}$ and $X_{m,xylene(2)}$ are significantly different
	3	7.65	0.26	96.6	$X_{m,A100(1)}$ and $X_{m,butanol(3)}$ are significantly different

	4	6.35	0.11	98.3	$X_{m,A100(1)}$ and $X_{m,butanol(2)}$ are significantly different
Ear	1	4.19	0.23	94.5	$X_{m,A100(1)}$ and $X_{m,xylene(2)}$ are significantly different
	2	4.32	0.93	78.5	$X_{m,A100(1)}$ and $X_{m,xylene(2)}$ are significantly different
	3	5.02	0.32	93.6	$X_{m,A100(1)}$ and $X_{m,butanol(3)}$ are significantly different
	4	3.76	0.08	97.9	$X_{m,A100(1)}$ and $X_{m,butanol(2)}$ are significantly different

5.2.5 Hematoxylin-eosin staining

Sections obtained from paraffin blocks containing tissue matrices and stained topographically with hematoxylin-eosin displayed visible differences between the four processing protocols (Figure 5.11). Depending on the organ processed, artifacts can be observed after staining, especially for ear, muscle and testis. Artifacts such as folding or even complete detachment of tissue sections from the slide are almost always evident for Protocol II and Protocol I (both using xylene as a clarifying agent). Significantly fewer artifacts were observed for Protocol III and Protocol IV, especially with regards to folded sections.

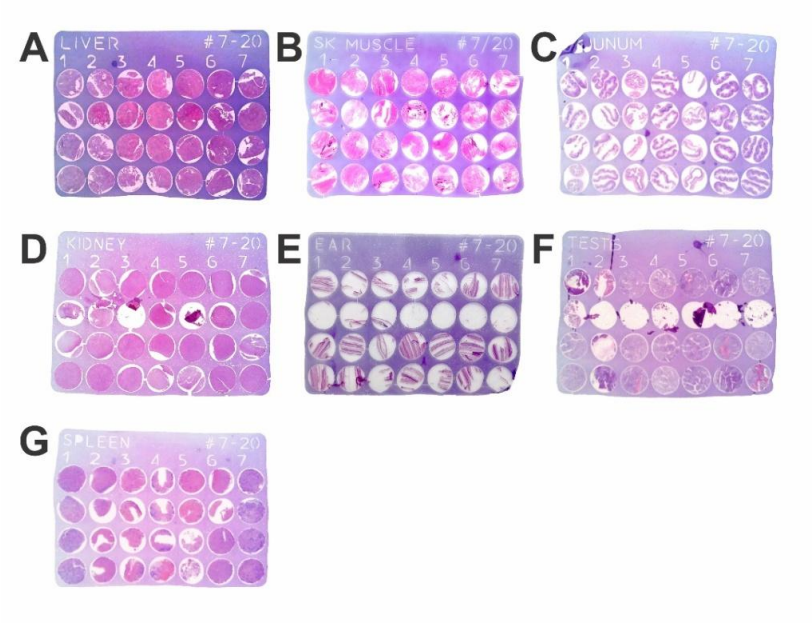

Figure 5.11. Hematoxylin-eosin staining. Tissue microarray containing the following tissues: (A) liver, (B) muscle, (C) intestine, (D) kidney, (E) ear, (F) testis, (G) spleen.). Lines 1 - 4 correspond to Protocols I – IV

5.2.6 Immunohistochemical staining

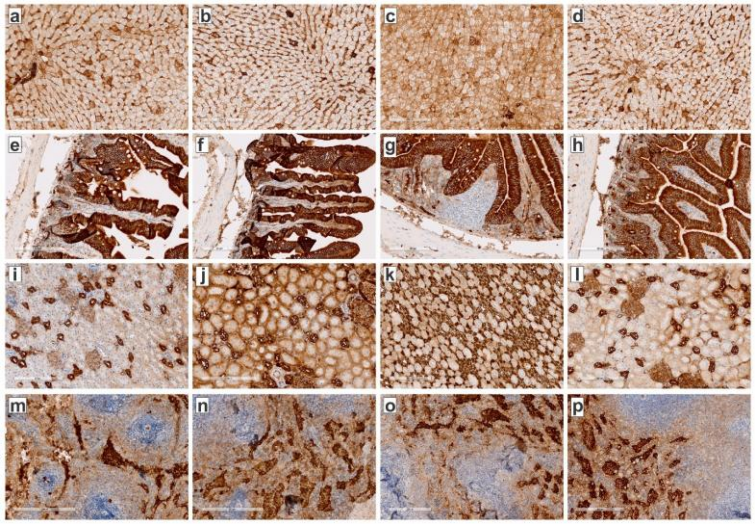

Figure 5.12 Immunohistochemical staining with horseradish peroxidase conjugated anti-cytokeratins AE1/AE3 antibodies: a) liver - processing I; b) liver - processing II; c) liver - processing III; d) liver - IV processing; e) intestine - processing I; f) intestine - processing II; g) intestine - processing III; h) intestine - IV processing; i) kidneys - processing I; j) kidneys - processing II; k) kidneys - processing III; l) kidneys - IV processing; m) spleen - processing I; n) spleen - processing II; o) spleen - processing III; p) spleen - processing IV. Scanning with 20x objective (Ocus 20, Grundium Oy, Tampere, Finland)

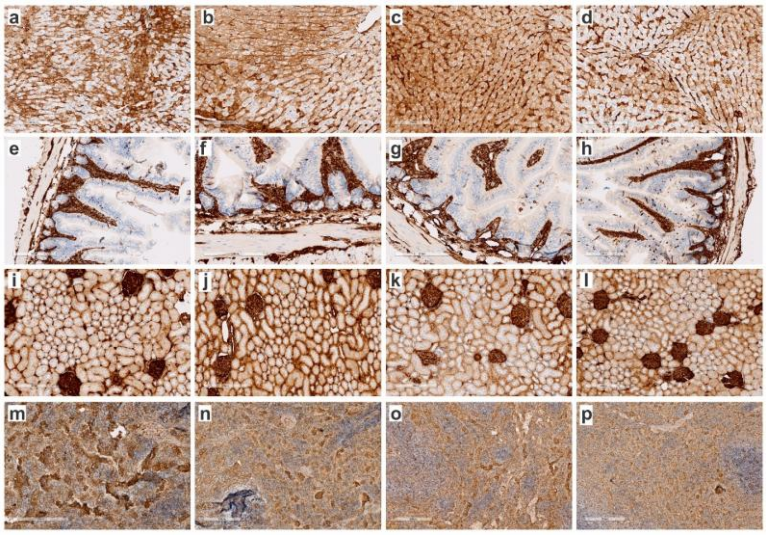

Figure 5.13. Immunohistochemical staining with horseradish peroxidase conjugated anti-vimentin antibodies: a) liver - processing I; b) liver - processing II; c) liver - processing III; d) liver - IV processing; e) intestine - processing I; f) intestine - processing II; g) intestine - processing III; h) intestine - IV processing; i) kidneys - processing I; j) kidneys - processing II; k) kidneys - processing III; l) kidneys - IV processing; m) spleen - processing I; n) spleen - processing II; o) spleen - processing III; p) spleen - processing IV. Scanning with 20x objective (Ocus 20, Grundium Oy, Tampere, Finland)

In Figures 5.12 and 5.13 it can be seen that the tissue samples stained specifically with both anti-vimentin and anti-cytokeratin AE1/AE3 antibodies, regardless of the processing protocol employed. However, with regards to non-specific, background staining, significant differences between the processing protocols performed could be observed: less non-specific staining and implicitly a higher optical contrast for room temperature protocols and when

using butanol as a transition solvent. Although a quantification of optical contrast is somewhat subjective, the order of optical clarity (from best to worst) appears to be butanol at RT, butanol at 37 °C, xylene at RT, xylene at 37 °C.

CHAPTER 6. THE INFLUENCE OF HISTOLOGICAL SPONGES DURING PROCESSING OF TISSUE SAMPLES

In histology laboratories, histology pads (sponges) are used to prevent the loss of small pieces of tissue during processing as well as for maintaining biopsy orientation. The degree of sponge porosity and carry-over/retention of fluids during histological processing is a neglected aspect until now and can be extremely variable, depending on the manufacturer. Due to the structure and absorption capacity of the materials from which the sponges are made, they can retain water and transport it from one solution to another, causing the change of the processing protocol by diluting the dehydration solutions. The use of “contaminated"/diluted solutions can make the processing protocol ineffective, and residual water may remain in the tissue. The inclusion in paraffin of tissues that still have traces of water as well as improper storage conditions (high humidity, fluctuating temperature, etc.) of the resulting paraffin blocks can adversely affect the quality of the stained tissue slides, favor hydrolysis and/or oxidation of protein biomarkers and nucleic acids, resulting in the loss of antigenicity, diminished reactivity, etc. [5, 52, 53]. Since there are very few studies regarding the influence of histological pads on tissue preservation within histological samples, we dedicated the next chapter on a detailed study of the effects of histological sponges on dehydration solutions and implicitly on the impact on the integrity of processed tissue. The study assessed the absorption/retention capacity ("carry-over") of three types of sponges used currently in histology laboratories; determination of the percentage of shrinking/contraction of sectionable matrices containing core tissue biopsies following various processing protocols and measurements of the concentration of ethanol within the holding reservoirs for the sequential solutions used by automatic tissue processors. The quality of the processing protocol was evaluated by both topographic hematoxylin-eosin and immunohistochemical staining of the tissues processed using the histological sponges under investigation.

In this study was used fresh pig tissue (kidney) from which multiple biopsies were collected using a biopsy gun (Bard Max-Core, Bard Biopsy Systems, Tempe, USA) using a 16 Gauge tru-cut needle (the diameter of the resulting biopsies is approximately 1.2 mm) and multiplexed in BxChip™ sectionable arrays with 6 groove shaped cavities (Figure 6.5). Three different types of sponge pads used routinely in histology laboratories were tested (Table 6.1): histological sponge A (Themis Pathology SRL/ LUMEA Inc., USA), histological sponge B (BioPad Biopsy Pads, CellPath Ltd., UK), histological sponge C (Biopsy Foam Pads, Simport® Scientific, Canada). Histological cassettes without sponges were used as control samples (Figure 6.5).

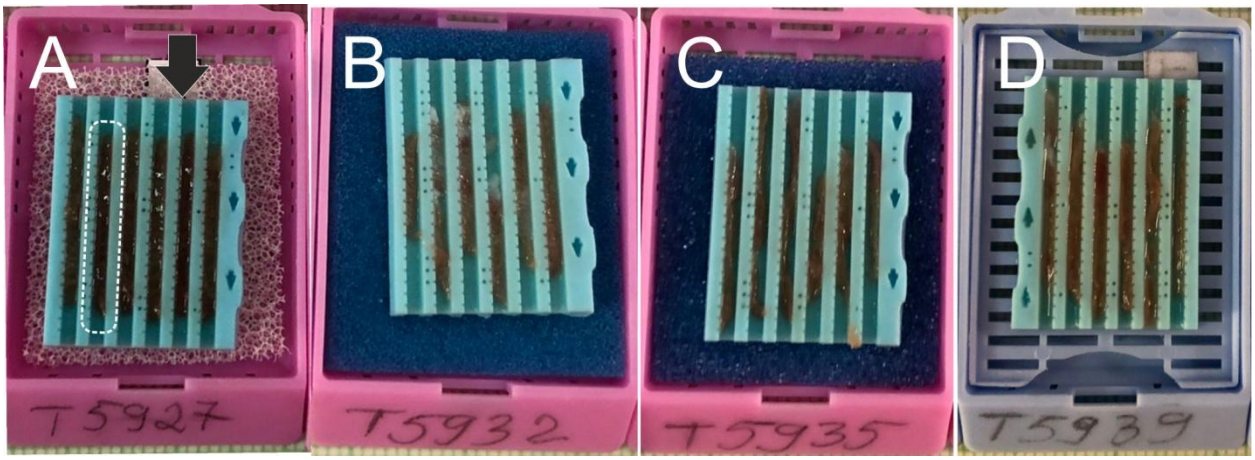

Figure 6.5. Cassettes with histological sponges: A: sponge Themis Pathology SRL/ LUMEA Inc., USA, B: sponge CellPath Ltd., C: sponge Simport® Scientific, D: histological cassettes without sponges. A 16 Gauge biopsy can be seen in the dotted white border, and the sectionable matrix (BxChip™) is highlighted with the black arrow.

Table 6.1. Physicochemical properties of the tested biopsy pads

Pad	Color	Composition	Dimension (mm) (length×width×thickness)	Weight (grams)	Porosity (Pores per Linear Inches/ppi)
A	White	Polyester polyurethane	3 × 2,5 × 3.3	1.5	40 - 50
B	Blue	Polyurethane	3 × 2.5 × 2.8	1.2	70 - 100
C	Blue	Polyester polyurethane	3 × 2.5 × 2.2	0.9	55 - 65

6.2.1. The absorption capacity and residual contamination ("carry-over") of the histological pads

The capacity of the three types of sponges tested to absorb and retain water is different. After the first step of removing water from the pores of the sponges, and draining them on a sieve, sponge B contains up to 35% water, while sponge A and sponge C have about 25%. After gentle centrifugation (150 rpm for 1minute) sponge B retains 30% water, sponge C 20%, and sponge A retains 5%.

6.2.2. The percentage of shrinking of the sectionable matrices according to the processing protocol

The BxChip™ matrix was measured (length and width) after each processing (see Table 6.3.) The values from the table represent the averages of four sectional matrices measurements for each processing protocol and the standard deviation of the average. The results show that matrices processed without histologically sponges shrieked the most. Starting from the initial size of 23 x 14 mm, after tissue processing it reaches 16.6 x 12.6 mm,

unlike the matrices processed with histological sponge B (which also retain the largest amount of water): which shrinks only 17.5 x 13.3 mm.

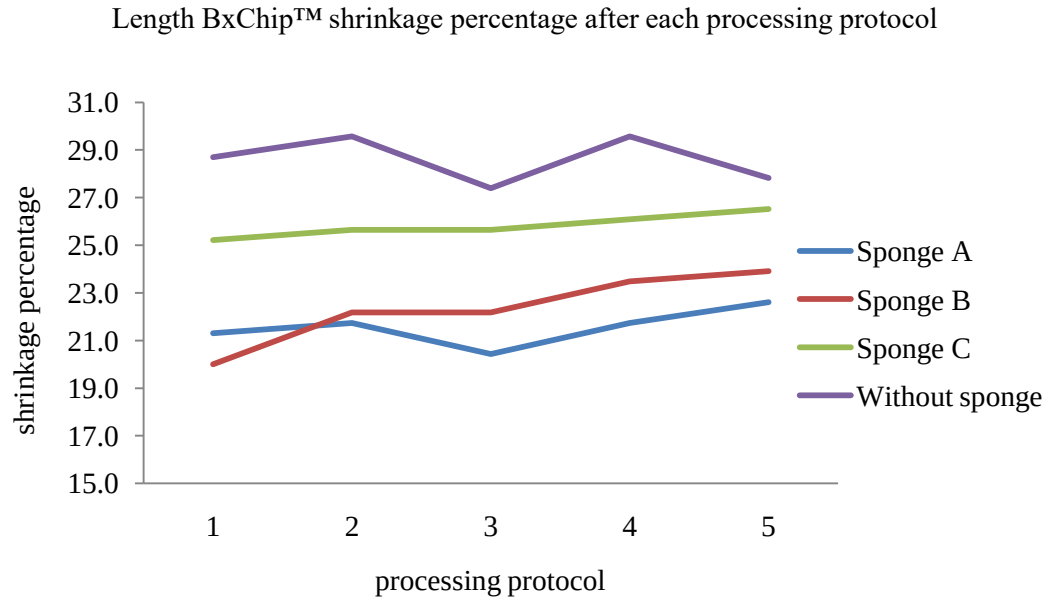

Figure 6.8. The graph highlights the percent shrinkage (length only) of the BxChip™ after each processing protocol. Values represent the average of four determinations.

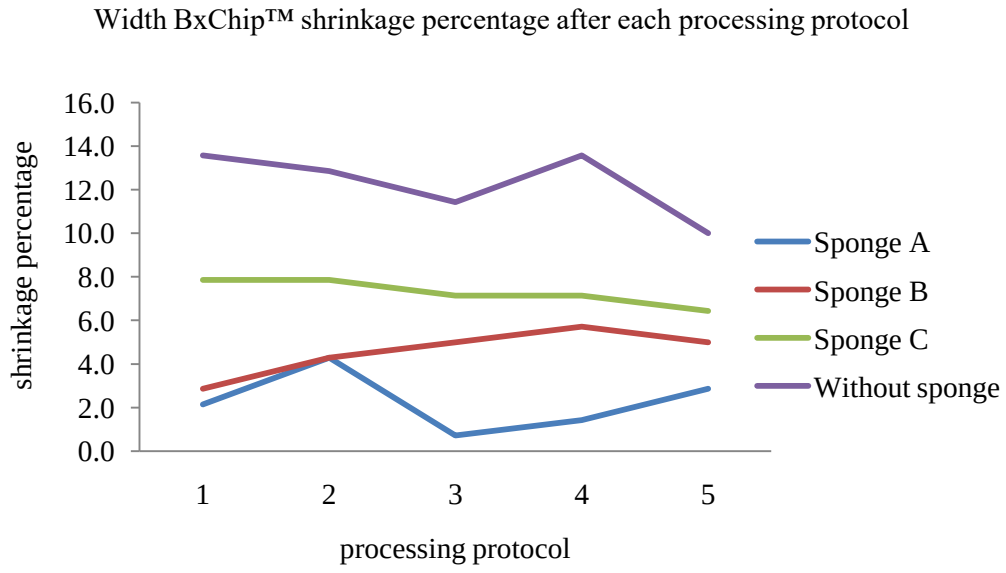

Figure 6.9. The graph highlights the percentage of BxChip™ shrinking in width after each processing protocol. Values represent the average of four determinations.

6.2.4. Gravimetric measurement of ethyl alcohol concentration in tissue processing solutions

Table 6.4. Ethanol concentration was determined using the online calculator (www.handymath.com) "Perry's Handbook for Chemical Engineers" (Robert H. Perry, Don Green, Seventh Edition). The alcohol temperature at the time of weighing was 20 °C, and the values represent the average of three determinations. Histological pads: A – Themis Pathology SRL/ LUMEA Inc, B – BioPad Biopsy

Pads, C – Biopsy Foam Pads, D – without histological sponge

Sponge	Dehydration step	Ethanol concentration					
		initial	after first processing	after the second processing	after the third processing	after the fourth processing	after the fifth processing
A	1	64.5	54.59	45.13	38.35	32.34	27.23
	2	95	92.56	87.34	82.89	78.16	73.32
	3	95	95.64	94.56	93.58	92.10	90.13
	4	95	96.46	95.40	95.53	94.49	94.23
	5	100	100.00	100.00	100.00	99.57	99.07
	6	100	100.00	100.00	100.00	100.00	100.00
	7	100	100.00	100.00	100.00	100.00	100.00
B	1	64.5	51.64	39.27	31.54	21.63	15.05
	2	95	88.47	79.76	71.21	64.48	55.53
	3	95	95.12	92.66	89.07	84.54	79.43
	4	95	95.78	94.95	93.90	92.64	91.01
	5	100	100.00	99.54	98.46	97.65	96.50
	6	100	100.00	100.00	100.00	100.00	99.29
	7	100	100.00	100.00	100.00	100.00	100.00
C	1	64.5	54.70	47.19	40.35	33.64	28.61
	2	95	90.47	83.97	78.03	71.27	65.39
	3	95	95.28	93.16	90.92	87.78	84.43
	4	95	95.87	94.93	94.16	93.19	92.22
	5	100	100.00	100.00	99.30	98.29	97.37
	6	100	100.00	100.00	100.00	100.00	99.82
	7	100	100.00	100.00	100.00	100.00	100.00
D	1	64.5	59.42	54.59	50.55	46.92	43.94
	2	95	93.59	90.99	88.41	85.57	83.10
	3	95	95.74	95.10	94.41	93.51	92.51
	4	95	95.98	95.38	95.16	94.49	94.01
	5	100	100.00	100.00	100.00	100.00	99.72
	6	100	100.00	100.00	100.00	100.00	100.00
	7	100	100.00	100.00	100.00	100.00	100.00

A detailed analysis of the first change of ethanol dehydration solution (Table 6.4) shows that after 5 processing cycles without renewing it (this is common practice in all histology laboratories) with the initial concentration of 64.5%, the actual concentration is lowered/degraded to 15% if sponge B is used and 27% for sponge A or C. The same trend was also observed for the following alcohol concentrations. An “equilibrium” was only seen with regards to the last alcohol change, where the concentration measured becomes the same with the desired value. However, for sponge A protocols, the ethanol concentration measurements are almost the same with the spongeless processing protocols. Due to the higher porosity (40 – 50 ppi) this type of sponge allows the passage of solvents much easier and the contamination of the solvents with water in the next steps of processing protocol is the smallest. The results in Table 6.4 confirm our initial measurements where we tested the “carry-over capacity” of the sponges: sponge A and sponge C retain less water and solvent, which allows for faster and more complete drainage of liquids.

6.2.6 Hematoxylin-eosin staining

Microscopic examination of tissue sections (Figure 6.16) reveals differences in the intensity

and contrast of the staining along the 5 successive processing protocols. The intensity of hematoxylin-eosin staining decreases as the amount of water in the processing solutions increases. For example, after processing 1 (the first alcohol had a concentration of 59%) the color is more intense (Figure 6.16a) than after the last processing (the first alcohol had a concentration of 44%) where it is uneven and less intense (Figure 6.16e). Also fragmentation of the biopsy core was observed, regardless of the processing protocol, if sponge A is used (Figure 6.16f-j). This is due to the rougher structure and increased pressure exerted on the biopsy by the somewhat thicker sponge. The most obvious degradation of stainability can be observed in the tissue samples processed with sponge B, where the intensity of the coloration drops dramatically, the biopsy becomes fragmenteds, and voids and folds of the tissue sample become apparent on the microslides (Figure 6.16p-t). These artefactual features seem to correlate with the lower concentration of ethanol in the processing solutions: after 5 succesive processings the first ethanol decreases to 15% (from the initial 64.5%) while the second ethanol solution goes down to 55.5% (from 95%, initially).

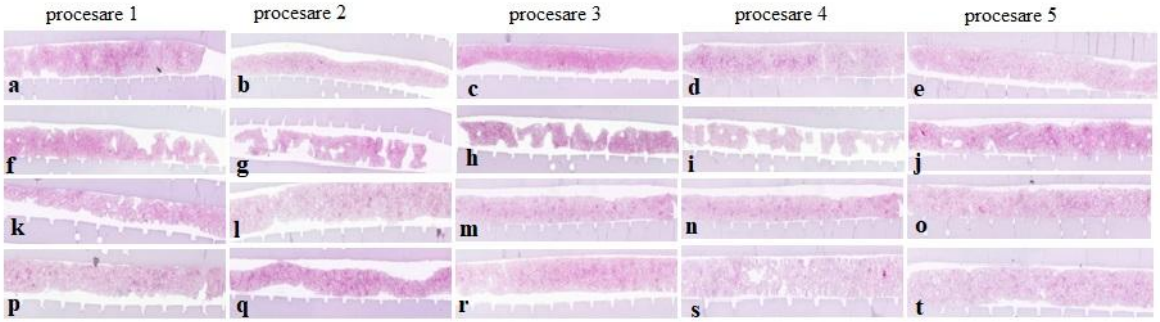

Figure 6.16. The impact of the histological sponge on the biopsy and on the intensity of hematoxylin-eosin staining. a-e: without histological sponge, f-j: sponge A, k-o: sponge C, p-t: sponge B. Scan with 20x objective (Ocus 20, Grundium Oy, Tampere, Finland)

6.2.7 Imunohistochemical staining

The overall conclusion is that the progressive changes of the processing protocol generated by the deterioration of the dehydration solutions (as a result of the histological sponges) significantly influences the intensity/quality and less the specificity of immunostaining, with potentially serious consequences on the accuracy of the anatomopathological diagnostic. When all the test conditions are compared (3 types of histological sponges, 4 processing protocols), the immunohistochemical expression of collagen IV decreases in intensity (especially regarding tubular basal membranes) paralleling the level of degradation of the solutions. This situation is more pronounced for sections generated from tissue samples processed with sponge B where the intensity of specific staining is minimal. In the very same samples a progressive increase in non-specific, background staining can be observed, which, coupled with the general decrease in the staining intensity of the positively identified structures, can lead to difficulty (up to impossibility) of accurate microscopic diagnosis. On the other hand, no intensity differences are observed regarding the expression of vimentin between the tissue samples tested, regardless of the type of sponge employed. It would be interesting to examine this artefactual behaviour of immunoreactivity as a function of the histology pad used during tissue processing for a larger selection of antigens, because it seems that some epitopes are more sensitive than others.

CHAPTER 7. THE INFLUENCE OF PRE-ANALYTICAL VARIABLES ON SECTION RETENTION DURING IMMUNOHISTOCHEMICAL STAINING

The influence of pre-analytical and analytical variables on immunohistochemistry analysis are closely related, meaning that a well-fixed and processed tissue will result in good quality and specific staining. Detachment of sections during this type of staining (typically more aggressive than the usual topographical stains) is an unwanted accident, sometimes with catastrophic consequences (irreversible loss of unique preparations, absolutely necessary for precision diagnosis) and may signal defects in the pre-analytical steps and/or operator negligence. In the present study, we aimed to analyze the influence of different pre-analytical variables on the accidental detachment of the paraffin sections from the microscope slide during immunohistochemical staining. The studied parameters were tissue type and biopsy size, fixation and processing protocols, and the type of antigen retrieval solution used for unmasking of immunoreactivity. The fixation and processing protocols tested in this study are the same protocols that were tested in Chapter 5; the present chapter (chapter 7) representing an extension of the initial experiment where we tested the influence of the fixation and processing protocols on the amount of residual water in the tissue and on the quality of immunohistochemical staining. In this chapter we will study in detail if the fixation and processing protocols have a significant role in the detachment of sections from the microscope slide during immunohistochemical staining. Fresh pig tissue was used for this study. The tissues tested were kidney, liver, spleen, tongue, pancreas, heart and stomach, from which 5 mm core biopsies were taken and subjected to various processing protocols. To avoid inter-experimental variability, all organs were harvested from a single animal. To reduce the costs

of this study we used tissue arrays to embed several biopsies in a single paraffin block (tissue microarrays - TMA). For comparison, 4 matrices with 1 mm cavities and 42 matrices with 3 mm cavities were tested (Figure 7.3.). All samples were manually processed according to three fixation protocols: fixation A: 264h at room temperature in 10% NBF; fixation B: 2h at 4 °C and 262h at room temperature in 10% NBF; fixation C: 12h at room temperature without fixation and 252h at room temperature in 10% NBF followed by 4 different processing protocols (tested in Chapter 5). After processing the samples, sectioning the paraffin blocks and affixing the sections on the microscope slide, the protocols for immunohistochemical staining and topographic staining with hematoxylin-eosin were performed.

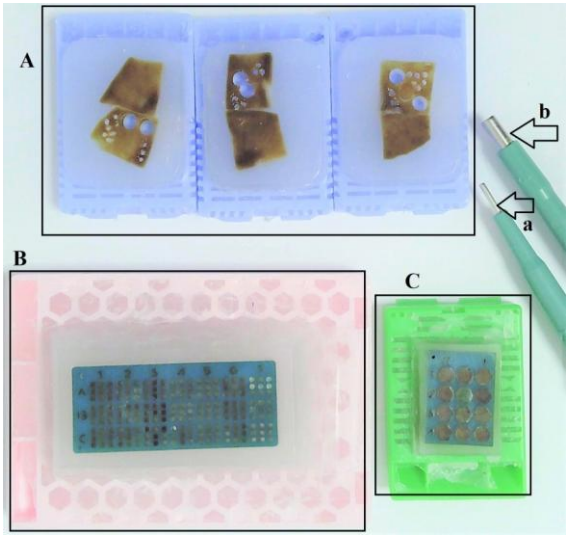

Figure 7.3. (A) "Donor" paraffin block with kidney biopsies, (B) "Recipient" paraffin block with TMA that has embedded 1 mm diameter kidney biopsies, (C) "Recipient" paraffin block with TMA that has embedded 3 mm diameter kidney biopsies, (a) 1 mm diameter dermatological punch, (b) 3 mm diameter dermatological punch

7.2. Results

Depending on the biopsy size, the percentage of section detachment or folding from the glass slide is different. As can be seen in Table 7.1, sections with biopsies of 1 mm diameter detached and folded the most (37% from a total number of 756 biopsies), regardless of the type of tissue, fixation protocol or the processing protocol.

Table 7.1. Percentage of section detachment depending on the biopsy size.

Biopsy size (mm)	Percentage	Percentage of section detachment/folding													
		Tissue							Fixation			Processing protocol			
		heart	kidney	liver	pancreas	spleen	stomach	tongue	A	B	C	I	II	III	IV
1	37	38	3	22	33	4	57	100	37	32	41	24	48	42	32
3	12	11	1	4	11	0	28	32	9	12	16	14	24	7	6
5	16	8	0	0	17	8	75	0	11	14	21	14	29	14	5

Tissue fixation should be performed as soon as possible to prevent tissue degradation due to the time that elapses between tissue sampling and fixation (cold ischemia). The results of the study show us that, regardless of the biopsy size, the biopsies where their fixation was delayed (the biopsies were left for 12h at room temperature without fixation and only then were they fixed at room temperature for 252h in 10% NBF) detach from the glass slide as follows (Table 7.1): 21% (5 mm biopsies), 16% (3 mm biopsies) and 41% (1 mm biopsies). The processing protocol (Table 7.1) also influences the percentage of sections showing folding of the sections: 29% of the sections that are processed with Protocol II (5h30 minutes at RT, clarification solution: xylene). The fewest folded sections (5%) are observed in Protocol IV (3 days fixation at RT, clarification solution: *n*-butanol). After testing different antigen retrieval solutions (Tris-EDTA buffer (pH=9); citrate buffer (pH=6), EDTA buffer (pH=8), we noticed that the biopsies that are lost the most (12%) are the biopsies treated with Tris-EDTA (pH=9) and regarding tissue type, the most unstable are the tongue (32%) and the stomach biopsies (28%).

CHAPTER 8. COST ANALYSIS IN THE HISTOLOGY LABORATORY: TMA VS CLASSICAL METHOD

The in vitro diagnostic medical devices developed in the Themis Pathology research laboratory (Voluntari, Ilfov, Romania) are based on a number of proprietary tissue microarray technologies and aim to multiplex tissue samples to obtain fast and economical results. For the present study, a support, sectionable matrix customized according to the number of samples to be analyzed was developed to allow the embedding, fusion and multiplexing of the tissue samples used in the experiments. In this chapter the method of obtaining sectionable matrices is described, followed by a tentative cost analysis of the implementation of this technology in the histological laboratory. The biomimetic material was purchased from the manufacturing company Themis Pathology S.R.L (Voluntari, Ilfov, Romania) and it was used for the generation of custom paraffinized tissue matrices. The properties of this material allowed fixation in 10% NBF, dehydration in increasing concentrations of ethanol, clarification with a transition solvent and infiltration with paraffin.

The resulting material was engraved with a Speedy 300 laser (Trotec Laser GmbH, Austria) in the desired shapes:

- Model 1: support (length 50 mm × width 37 mm × thickness 2 mm) with 28 round cavities with a diameter of 6 mm created by laser cutting (into which the tissues will be inserted). The resulting matrices were used in the study from Chapter 5.

- Model 2: a support with dimensions of 22 × 15 × 2 mm, and 12 hexagonal cavities with a diameter of 3 mm. The resulting matrices were used in the study from Chapter 7.

- Model 3 (41 × 18 × 2 mm) with 189 round cavities with a diameter of 1 mm. The resulting matrices were used in the study from Chapter 7.

For cost analysis, the labor and consumables used after processing of the samples were considered to obtain the conventional blocks compared to the "recipient" blocks containing the tissue matrices. The study includes the costs that occur after the preparation of the "donor" blocks: the cost of the customized matrix, all the consumables used to obtain the slides from the "donor" and "recipient" block and the time required by the histotechnologist to obtain the slides stained with hematoxylin-eosin. The cost of the matrix is the total cost and includes the biomimetic material, the solutions used for processing and laser engraving. The cost also includes laboratory expenses (based on 2022 average market prices of supplies and 2022 wages) but does not include the time the pathologist spends analyzing the slides.

Depending on the organ and pathology, reading the slides can take anywhere from a few seconds when the pathologist is looking for the presence of a marker, to a few minutes when looking for discrete cell abnormalities.

Tabel 8.8. The total cost of histopathology services: conventional versus multiplexing

Stage	Model 1		Model 2		Model 3	
	Conventional 196 blocks	7 TMA x 28 cavities	Conventional 504 blocks	42 TMA x 12 cavities	Conventional 756 blocks	4 TMA x 189 cavities
TOTAL CONSUMABLE COST	380.6	135.21	1646.48	764.44	2419.92	84.85
TOTAL LABOR COST	356.39	82.15	916	354	1375	267
TOTAL COST (RON)	736.99	217.37	2562.90	1118.51	3794.55	352.20
Percentage decrease	71		56		91	

The multiplexing method significantly speeds up the preparation and examination of histological preparations and at a lower cost, because it allows the simultaneous analysis of several samples on a single slide. The cost reduction in the histology laboratory when sectionable tissue arrays are used is considerable. Depending on the model used, the total cost of histological services can be reduced by up to 91% (Table 8.8). Even though the transfer of biopsies from the "donor" blocks to the "recipient" blocks is a step that requires great care and very good sample traceability, the receiving paraffin block containing a tissue matrix with a predefined design ensures superior traceability, and the blocks can be stored for long periods for subsequent validations. Last but not least, a very important aspect is the fact that the use of TMA in diagnostic laboratories makes possible to reduce the storage space of paraffin blocks and histological slides, a real problem at the moment in all laboratories and countries of the world, because the legislation requires their safe keeping between 20 and 30 years.

CHAPTER 9. IMPLEMENTATION OF THE *n*-BUTANOL PROCESSING PROTOCOL IN PRECLINICAL TESTS REQUIRING MORPHOPATHOLOGICAL EXAMINATION

The preclinical component of the paper presents the results obtained in two preclinical studies in which the processing protocol that provided the best results (*n*-butanol protocol, 3 days protocol at room temperature) and tissue matrices (TMA) was implemented. Processing protocol IV is carried out at room temperature for 61 h, uses *n*-butanol as a clarifying agent and provides the best results in terms of the amount of residual water left in the processed, paraffin infiltrated tissue sample and implicitly better topographic and immunohistochemical staining. The first study was conducted to test two non-steroidal anti-inflammatory drugs (NSAIDs) and the second study to test products from the Imunoinstant range (Romvac Company SA, Voluntari, Romania), which represents a source of antibodies for passive immunotherapy. For both studies, tissue arrays were used for multiplexing the samples in order to ensure the traceability of the samples. At the same time, the use of matrices helps to reduce the costs in the histology laboratory, reduce reading time of the slides and decrease inter-experimental variability regarding immunostaining. The first study (testing anti-inflammatory drugs in an experimental model of psoriasis) was done on mice, applying a cream with these drugs on the surface of the tail for two weeks.

Psoriasis is a disease characterized by the reduction or disappearance of the granular layer of the skin following the aberrant differentiation of keratinocytes. Non-steroidal drugs with anti-inflammatory effect are used in dermatology due to their inhibitory effect on the enzyme cyclooxygenase-2 (with a role in stimulating the aberrant proliferation of keratinocytes) [115, 116]. The NSAIDs used in this study are diclofenac (2-[2-(2,6-dichloroanilino) phenyl] acetic acid) and celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl) pyrazole-1] benzenesulfonamide) in different concentrations, to see if a greater inhibition of cyclooxygenase-2 would mean an increase in the epidermal granular layer [115, 116, 117, 118].

The tests were carried out in collaboration with the Pharmacology and Pharmacotherapy Department, "Carol Davila" Faculty of Medicine in Bucharest. The second study consisted of immunohistological investigations in C57BL/6 mice, which were orally administered hyperimmune egg emulsion and immunoglobulin Y complexes. These products were generated by SPF chickens treated with a specially prepared antigen mixtures and contain immunoglobulin Y, lysozyme, ovotransferrin etc. [119, 120]. The products developed and marketed by Romvac Company S.A under the IMUNOINSTANT brand tested in this study: Hyperimmune Egg, Imunoinstant G, Imunoinstant multiple (aqueous solution).

9.2. Results

Study 1:

Table 9.2 shows a significant difference in the degree of orthokeratosis between the negative control group (untreated mice) and the groups treated with celecoxib, diclofenac and tretinoin (positive control). Different concentrations of celecoxib lead to an increase in the degree of orthokeratosis in direct relation to the concentration of the drug. The induction of

epidermal differentiation, as a marker of the degree of orthokeratosis, was in the following order: celecoxib 8 % > celecoxib 4 % > celecoxib 2 % > tretinoin 0.05 % > diclofenac 1 % > celecoxib 1 % > diclofenac 2 % > soft white paraffin > untreated mice. The effect of celecoxib 8 % on the degree of orthokeratosis is superior to that of tretinoin. Percent drug activity was highest for celecoxib 8 % (70.59 %) (see Table 9.2).

Table 9.2. The effect of the tested substances is expressed as the degree of orthokeratosis (percent), the average thickness of the epidermis (μm) and the percentage activity of the drug.

Mice group	**Degree of orthokeratosis (%)**	**The average thickness of the epidermis (μm)**	**Drug activity (%)**
Untreated mice	17.7 ± 1.81	24.77 ± 1.81	-
Soft white paraffin	19.64 ± 3.2	29.76 ± 2.02	0
Tretinoin 0.05%	48.72 ± 6.09	33.22 ± 2.1	36.19
Diclofenac 1%	42.24 ± 13.73	28.42 ± 2.62	28.12
Diclofenac 2%	41.42 ± 8.17	27.38 ± 2.75	27.1
Celecoxib 1%	42.15 ± 7.28	28.07 ± 2.37	28.01
Celecoxib 2%	56.48 ± 11.33	32.28 ± 6.18	45.84
Celecoxib 4%	71.64 ± 3.43	31.66 ± 3.58	66.56
Celecoxib 8%	75.06 ± 3.37	33.43 ± 3.69	70.59

Protocol IV (which uses *n*-butanol as a clearing agent) helps to obtain high-quality, time-stable histological blocks (no depressions are formed on the surface of the block, the tissue is completely fused with paraffin, trouble-free sectioning, good quality ribbons generated during sectioning, high retention rate of the tissue sections on the glass slide regardless of the histological staining performed, etc.). This processing protocol was selected for facilitating an easy and precise measurement of the granular layer (*i.e.* degree of orthokeratosis).

Study 2:

The results of the study showed that the products from the Imunoinstant range that contain immunoglobulin Y are effective and induce an immune response depending on the type of product, dose and time of administration. the analysis performed at different times of collection of serum samples (T1 – 30 days, T2 – 60 days, T3 – 90 days) showed a significant increase in the concentration of interleukins, which have the main role of activating effector cells during inflammatory and immune responses.

After 30 days of supplement administration, an increase in the following interleukins is observed:

Group A: IL-4, IL-5, IL-9, IL-13, IL-17A, GM-CFS, IFN-G, TNFα.

Group B: IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IL- 12, G-CFS, GM-CFS, TNFα, Eotaxin, IFN-G, MIP-1°, IL-33.
Group C: IL-10, Eotaxin, IL-23, IL-33.
Group D: IL2, IL-3, IL-10, IL-12, IL-17A, Eotaxin, IL-17F, IL-25, IL-27, IL-31, IL-23, IL-33.

After 60 days of supplement administration, an increase in the following interleukins is observed:
Group A: TNFα.
Group B: IL-2, MIP-1a, Rantes, IL-22, IL-31.
Group C: IL-17A, GM – CFS, IFN-G, MIP-1a, Rantes, IL-22, IL-31.
Group D: Rantes.

After 90 days of supplement administration, an increase in the following interleukins is observed:
Group A: IL-17A, Eotaxin, GM-CFS, IFN-G, IL-22. L.
Group B: IL-2, IL-10, IL- 12, IL-13, IL-17A, G-CFS, GM-CFS, TNFα, IFN-G, IL-25, IL-27.
Group C: IL-2, IL-3, IL-4, IL-9, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, G-CFS, GM-CFS, TNFα, IFN-G, IL-25, IL-22, IL-27.
Group D: IL-10, IL-22, IL-17F, IL-27, IL-31, MIP-3a.

Protocol IV (which uses *n*-butanol as a clarifying agent) helps to obtain good histological blocks that retain their properties over time. Even after a few months after their sectioning and exposure to the laboratory environment (22 ± 2 °C, relative humidity of 50 – 70 %), no depressions are observed on the surface of the block. The tissue fused very well with the paraffin and sectioning with the microtome is without difficulty, ribbons of sections are quickly obtained, which allow their immediate application on the glass slide, and after staining with hematoxylin-eosin all the tissues remain on the slide without detachement and without folding. The use of sectionable matrices and their customization for the present study represented a real help in multiplexing the multitude of samples because it reduced the expenses with the consumables used, significantly reduced the time needed to prepare the materials and the histological stainings and virtually eliminated interexperimental variability by making posible immunostaining of a very large number of individual samples in one single batch.

GENERAL CONCLUSIONS

The doctoral thesis proposed both the investigation of some pre-analytical variables (tissue type, fixation protocol, processing protocol, histological sponges) on the quality of tissue samples for Pathological Anatomy and the development of an optimal tissue processing protocol which preserves optimally its structure and molecular composition. For this purpose, four major research directions were pursued:

1. *Determination of residual water left within the histology preparations (from the tissue samples themselves and/or from the processing solutions employed).*

The studies carried out led to the following conclusions:

- The residual water in the processed tissue samples can be reduced significantly by using a clarifying agent miscible with both water and paraffin (universal solvents such as *n*-butanol);

- Better quality paraffin blocks were obtained using a longer protocol with *n*-butanol to complete tissue dehydration;

- The protocol with *n*-butanol can be used even for the processing "capricious" tissues (testicular tissue);

- The protocol using *n*-butanol as a clarifying agent gives the best results in terms of the minimum amount of residual water in the tissue sample, resulting also in better morphological and immunohistochemical staining results;

- The results obtained from thermogravimetric analysis showed that samples that were processed using *n*-butanol as a clarifying agent contain the smallest amounts of residual water: 0.011 ± 0.001% vs 0.04 ± 0.004%, when clarifying with xylene ($p < 0.05$, values are significant).

- Statistical analysis confirm that Processing Protocol IV (with *n*-butanol) gives the best results in terms of tissue water extraction: the processing protocol and tissue water extraction parameters (solvent type/concentration and extraction stage number) had a significant effect ($p < 0.05$) on the water mass ratio;

- Inferior infiltration of paraffin into the tissue processed with protocols I and II (with xylene) was confirmed by the artifacts that appeared during hematoxylin-eosin staining: the tissue frequently detached from the microscope slide or folded during the staining protocol;

- The present study demonstrates that *n*-butanol can be used as a clarifying agent instead of xylene, because it continues to extract the last traces of water from the tissue throughout the clarification stage.

2. *The influence of histological sponges during tissue sample processing protocols*

Within this direction of research, three types of histological sponges that are commonly used in histology laboratories were comparatively investigated. The studies carried out led to the following conclusions:

- The histological sponge used during the biopsies fixation and processing protocols had a major influence on the degradation/contamination of the solutions used as well as on the tissue samples;

- Absorptive capacity and water retention of the sponges used in histology is determined by their porosity and structure (degree of reticulation). Histological sponges with high porosity and implicitly smaller pores (70 – 100 ppi) created the most problems, transporting water from the fixative solution and tissue from one container to another during processing;

- Sponge A (40 – 50 ppi) and sponge C (55 – 65 ppi) retain less water and solvent (5% and 20%, respectively) and drain liquids from their pores faster, unlike sponge B (70 – 100 ppi) which retains up to 30% water;

- Using the same solutions throughout several processing protocols causes a significant change in their concentration due to water carry over from both the tissue samples, but also via the histological sponges, effectively causing a change in the protocol itself. If initially the concentration of the first alcohol was 64.5%, as planned, after 5 days of processing with the same alcohol, it can decrease as low as 15%, when using histological sponge B. Under these

circumstances, in effect, each round of tissue processing will employ in reality a different protocol, further and further diverging from the intended procedure and generating worse and worse results regarding the staining and the morphology of the processed tissues;

- As the processing solutions degrade, the sections become paler and nuclear details are lost;

- The intensity of hematoxylin-eosin staining decreases as the amount of water in the processing solutions increases. For example, after the first processing (59% concentration in the first alcohol) staining is slightly more intense than after the last processing (44% concentration in the first alcohol) in the case of biopsies processed without a histological sponge. However, this degradation of tinctorial affinity (lower staining intensity, but also unevenness of staining) is more pronounced and appears as early as the second round of tissue processing when the biopsies were processed with a histological sponge;

- Changing the processing protocol significantly influences the quality and specificity of immunohistochemical staining, with potentially serious consequences for the accuracy of the anatomopathological diagnosis.

3. Section retention on the glass slides

In the present study, we sought to analyze the influence of different pre-analytical variables on the accidental detachment of the paraffin sections from the microscope slide during immunohistochemical staining. The studied parameters were tissue type and biopsy size, fixation and processing protocols, and antigen retrieval solutions. The studies carried out led to the following conclusions:

- Fixation and processing protocols must be adapted to the type of tissue. Stomach and tongue sections detached in the highest proportion, regardless of biopsy size;

- Tissue fixation should be performed as soon as possible to prevent tissue degradation due to the time that elapses between tissue sampling and fixation. The results of the study show us that, regardless of the size of the tissue fragment, if fixation was delayed (the biopsies were left for 12h at room temperature without fixation and only then were they fixed at room temperature for 252h in 10% NBF) the resulting paraffin sections can frequently detach from the glass slide, as follows: 21% (5 mm biopsies), 16% (3 mm biopsies) and 41% (1 mm biopsies);

- The unsatisfactory results obtained in the case of samples processed with protocol II (room temperature protocol of 5 hours and 30 minutes, and using xylene as a clarifying agent) are suggesting that the tissue is not adequately dehydrated and infiltrated with paraffin, causing the sections to detach from the supporting glass slide when harsher immunohistochemical protocols are applied. Using *n*-butanol instead of xylene (Protocols III and IV) facilitates continuation/completion of dehydration during the clarification step and results in better quality paraffin blocks and superior adhesion and resilience of the paraffin sections on the microscope slide;

- The experimental data presented in this chapter are strongly suggesting that inappropriate processing protocols are the main cause of section detachment from the glass slide, as follows: 29% (5 mm biopsies), 24% (3 mm biopsies) and 48% (1 mm biopsies).

4. Cost analysis in the histology laboratory: TMA vs the classical method

In the present study, a support sectionable matrix was developed that allowed the embedding, fusion and multiplexing of tissue samples. The matrix was customized according to the number of samples to be analyzed, and used throughout the experiments presented in this thesis. In order to analyze the effectiveness of this method in the histology laboratory, a cost analysis was made, and the study led to the following conclusions:

- The multiplexing method significantly speeds up the preparation and examination of histological preparations, and at a lower cost, because it allows the simultaneous analysis of several samples on a single slide;

- Cost reduction in the histology laboratory when sectionable tissue arrays are used is considerable. Depending on the model used, the total cost of histological services can be reduced by up to 91%.

- The recipient tissue matrix with a predefined design ensures superior traceability, and the blocks can be stored for long periods for later validations;

- The use of TMA in diagnostic laboratories makes possible a significant reduction of the storage space for paraffin blocks and histological slides.

ORIGINAL CONTRIBUTIONS

The general objective of the doctoral thesis was the physico-chemical study of tissue samples and the impact of pre-analytical variables in Pathological Anatomy. A series of original elements of this doctoral thesis are presented below:

1. Determining the water content of the tissue sample at different stages of processing, by equilibrating the samples with universal solvents and determining the amount of water in the solvents;

2. Determination of the fluctuating water content in the paraffined tissue by exposing it to moisture/drying cycles and weighing it with a high-precision analytical balance;

3. Determination of the water content of paraffinized tissues by thermogravimetry;

4. Quantification of water extraction from the tissue in different stages of processing;

5. Highlighting the fact that the clarifying agent used in the processing protocol has a prominent effect on the amount of water remaining in the tissue sample. *n*-butanol is miscible with both water and paraffin and, as such completes the dehydration stage while in the same time prepares the tissue for infiltration with paraffin, offering the best results;

6. Demonstrated for the first time the deleterious effects of histological sponges on the processing solutions and implicitly on the tissue samples, through the transport of water from one solution to another;

7. Tissue processing inconsistencies can negatively influence topographical or immunohistochemical staining, potential altering accuracy of diagnosis;

8. Demonstration that when inappropriate fixation and processing protocols are used sections can detach from the glass slide;

9. Proposing personalized tissue microarrays from paraffin-embedded samples as a diagnostic tool;

10. Cost analysis in the histology laboratory when custom sectionable tissue arrays are used. Depending on the model used, the total cost of histological services can be reduced by up to 91%.

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DISSEMINATION OF THE RESULTS

The results of the doctoral thesis were disseminated by presenting at an international conference, The results of the doctoral thesis were disseminated by presentation at an international conference, publication of 8 articles in ISI listed journals and 2 indexed articles and one article submitted for publication.publication of 8 articles in ISI listed journals and 2 indexed articles and one article submitted for publication.

Articles

1. Ștefan A. E., **Gologan D.,** Leavitt M. O., Mușat S., Pleșea I. E., Stan R., Pleșea R. M., Militaru M. **2020**. *Tissue MicroArrays, brief history, techniques and clinical future*. Romanian Journal of Morphology & Embryology, 61(4), 1077–1083. **[FI_{2020} = 1.033]**

2. Ștefan A. E., **Gologan D.,** Mușat S., Stan R., Sanda C. A., Militaru M. **2021**. *Biomimetic matrices for histological processing*. Revista Română de Medicină Veterinară, 31(3), 12–16. **[FI = 0]**
3. **Gologan D**., Ștefan A. E., Stan R., Militaru M., Mușat S. **2021**. *Factors influencing the quality of formaldehyde fixed paraffin embedded tissue samples – Review*. Revista Română de Medicină Veterinară, 31(4), 87–92. **[FI = 0.2]**
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