UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA

DOCTORAL SCHOOL

Ph.D.THESIS
ABNORMALITIES OF HAEMOSTASIS IN ISCHEMIC STROKE
ABSTRACT

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CONTENT

INTRODUCTION

Ischemic stroke is first in terms of all neurological diseases encountered in adulthood because of its frequency and importance. [1]. Activation of haemostasis components at the endoluminal surface of the atheromatous plaque occurs as a result of local lesions and / or the influence of local or systemic haemodynamic factors.

Central nervous system has certain structural and functional features that give it a higher degree of protection against hemorrhages, these particularities being mainly generated by the junctions between the endothelial cells of the small blood vessels and the under-expression of some antithrombotic molecules, thus in certain situations, thrombosis is favored.

Recently, the evaluation of coagulation by elastography in optical coherence, using acoustic radiation, in combination with OCT has been proposed. Thus, simultaneous changes in the elastic properties of the clot and the dynamic blood clotting status are evaluated. Haemostasis events that may be relevant from clinical point of view, are: reaction time, kinetic clot formation and shearing. This technique has the potential for in vivo application in the evaluation of hyper / hipocoagulability states or for treatment monitoring with pro or anticoagulants [5].

OCT may also become an in vitro study mode of the ischemic stroke thrombotic process. This is a non-invasive imaging technique that uses interferometric optics for performing a tomographic scan. Low coherence interferometry produces a two-dimensional image of biological microstructures with resolution (lateral and longitudinal) and increased sensitivity but with low penetration [6].

Blood clotting can also be tracked and quantified through the OCT because local changes in the optical properties of the blood - sputtering coefficient, refractive index, etc. appear in this process.

The purpose of this study is to dynamically assess the changes in the optic properties that occur in coagulating blood in a group of patients with ischemic stroke compared to a group of clinically healthy subjects.

From the data meta-analysis on the evaluation of the clott formation dynamics and its biophysical qualities, which we performed in this study, we found that this topic is still a scientific approach at the beginning. OCT studies on the coagulation process are only at the stage of trying to identify a dynamic and optical parameters that characterize physiological clotting

Key words: Ischemic stroke, Optical Coherence Tomography, coagulation, hypercoagulability, thrombosis.

PART I - THE STAGE OF KNOWLEDGE

This part contains three chapters:

1. ***Ischemic stroke****:* we documented the importance of the adressed problem, the ischemic stroke pathophysiology, the characteristic hemodynamics in this pathology, the mechanisms of neuronal death, the behavior of non-neuronal cells and white matter in ischemia, inflammatory events triggered, endogenous neuroprotection mechanisms and plasticity may coexist and may limit brain tissue destruction, therapeutic reperfusion outlook

2. ***Atheroslerosis, haemostasis and ischemic stroke*****:** in which we highlighted the events leading to this systemic illness but with focal symptoms, whose clinical manifestations are included in cerebrovascular disease, ischemic heart disease and peripheral arterial disease [109]. We also

developed activation of haemostasis components in atherosclerosis and associated vascular pathology, highlighting the structural and functional features of the brain in haemostasis and thrombosis.

3. Optical coherence tomography in the haemostasis – in this chapter we retained the relevant data on the method, we also did a meta –analysis of the OCT applications in medicine in general and in coagulation study in particular. capitol în care am reţinut datele relevante despre metodă, am făcut o metaanaliză a aplicațiilor OCT în medicină, în general şi în studiul coagulării, în particular. We have also highlighted the advantages and limitations of using OCT in the physiological and pathological haemostasis study.

PART II. SPECIAL PART - PERSONAL CONTRIBUTIONS

4.1. *WORKING HYPOTHESIS*.

Although haemostasis is a natural process by which the human body attempts to maintain a complicated balance between procoagulant and anticoagulant components, the failure of physiological haemostatic mechanisms has severe repercussions on human life and health. I refer to the two extreme pathological conditions of haemostasis, namely hipocoagulability and hypercoagulability.

Today, modern medicine successfully solves most pathologies due to deficiency of coagulant factors, while procoagulant status and vascular pathology associated with it are still far from a satisfactory medical solution. Procoagulant status (hypercoagulability) is associated with vascular pathology such as ischemic stroke, IMA, deep vein thromboembolism, etc., entities that represent a major vital danger that can be followed by disabling sequelae, the cost of which is burdened by health insurance systems, family and the community.

Exploration of haemostasis, at least at the level of medical practice, has been viewed and is still viewed formally, the many and often useful temporal tests serving to accumulate valuable data for diagnosis and treatment. Often, however, the exclusive use of such tests has led to the stagnation of research and its translation to other areas that may be of at least equal importance to biology, in general and to medicine in particular. I refer here, especially to the lack of correlation between temporal tests, sometimes with very wide limits, and the biological, functional value of the resulting fibrin clot, whose biophysical parameters are rarely discussed and much less quantified. The formation of the fibrin network is still seen by most researchers in a simplified way, according to the relationship: a coagulation test, regardless of which substrate is plasma or whole blood, is normal in relation to coagulation time.

Such a view is often denied by medical practice in which patients with normal coagulation times can develop a rapid and severe pathology of coagulation, either in minus-hipocoagulability or in addition in hypercoagulability.

Taking this evidence as a starting point, this study aims to treat this problem by a different approach and to broaden the exploration of haemostasis by investigating non-temporal parameters of the fibrin network, following dynamically the transformation of blood from the liquid phase into the solid with a light beam scanning a blood sample that coagulates. The method that allows such an approach is called optical coherence tomography (OCT), a method that allows the evaluation of the optic properties of the blood during the formation of the fibrin network.

4.2. STUDY PURPOSE AND OBJECTIVE

The purpose of this study is to dynamically assess the changes in optical coherence that occur in coagulation blood in a group of patients with ischemic stroke compared to a group of clinically healthy subjects. We have chosen this pathology model for two reasons: 1. the abundance of data from literature confirming the existence of a prothrombogenic status in patients with vascular accidents; 2. although exhaustive, these data do not answer questions about the quality of the formed clot: it is effective, it is aggressive for the endothelium, is too adherent or too lax, what physical properties are

involved, which are altered? Moreover, from the data meta-analysis on the evaluation of the dynamics of the coagulum formation and its biophysical qualities, which we made in this study, we have found that this topic is still a scientific approach at the beginning. Studies by the OCT on the coagulation process are only at the stage in which it is attempted to identify a dynamic and optical parameters that characterize physiological clotting.

The present study attempts to carry out coherent ophthalmic tomography in the area of cerebral ischemic vascular events, where for many years there have been reports of possible alterations of haemostasis without any certainty in this regard. The objectives of the study are: 1. description of the blood coagulation dynamics during OCT acquisition in a group of stroke patients compared to a control group; 2. verifying the hypothesis that in the blood of patients with ischemic stroke the OCT parameters could be altered as a result of the change in the biophysical properties of the fibrin clot; 3. identifying possible abnormal behaviors of coagulation dynamics during OCT to allow us to consider that such anomalies may constitute a reasonable marker of the thrombosis process and possibly the risk of thrombosis; 4. establish possible correlations that exist between the various parameters explored in this study.

4.3. MATERIAL AND METHOD

We conducted an observational descriptive study. We investigated blood clotting dynamics in patients with ischemic stroke, the blood samples were taken within 2 hours after the onset of neurological symptoms and hospital presentation, by using OCT and time-based methods for measuring coagulation parameters. We used classical tests for coagulation in order to evaluate haemostasis for several reasons: to determine whether the method we propose has a degree of specificity in studying haemostasis in this vascular pathology; to determine whether there are correlations between the optical parameters that form the fibrin network during coagulation and the clotting times investigated.

A group represented by a total number of **61** Caucasian patients with ischemic stroke was studied, of which 28 were women (45.90%) and 33 were men (54.09%), with an average age of 72.44 ± 8.48 years and a control group consisting of **18** healthy subjects, without stroke, without haemostasis abnormalities. In this group, the average age was 67.89 ± 11.21, and the gender distribution was the following: 10 women (55.56%) and 8 men (44.44%). Overall, the number of subjects included in this study was 79. Finally, depending on the different clinical and paraclinical characteristics of the patients enrolled in the study, the initial group of patients was subdivided into subgroups, that were considered at the time of statistical processing: ***Stroke Group*** -. patients with ischemic stroke, ***Stroke and Diabetes*** group - patients with ischemic stroke and type 2 diabetes, the ***Control Group*** - control sample.

We conducted this study taking into account the ethical and deontological principles of the Helsinki Declaration of Human Rights. Each subject was informed about the purpose and method of the study and expressed its consent to voluntary participation.

4.4. METHOD

For a good assessment of both the health status of the subjects included in the control group, and also of the vascular pathology or complications that patients with stroke - diabetes mellitus present, a working protocol was conducted in order to allow the collection of clinical and paraclinical data used in the study.

OCT images. Parameters analyzed..

Coagulated blood serum images were obtained with a OCT - OCT1300SS system, Thorlabs from the Coherent Optical Tomography Laboratory (Figure 4.3). The calibration of the system, the degree of mediation and the method of working with this system were carefully supervised by Professor Eugen Osiac, one of the promoters of this technique on biological tissues in Romania.

The OCT detection module is a CCD camera. The images obtained had a thickness of 0.5 mm and a depth of 0.5 mm. 3D scans were performed over a 0.5 mm length. This produces an image with a volume of 512x512x512 pixels. The final images were obtained as pixels' mean. The same parameters for image acquisition were used for all processed samples. The OCT acquisition was discontinued at the beginning of the experiment at 4, 9, and 15 minutes each time for 30 seconds. All images were analyzed with the IMAGEJ software. Image sets have been converted to stacks without any previous processing. Background noise was lowered by using an image processing algorithm. *The mean, the integrated density, the asymmetry and vaulting coefficients of the probabilistic distribution of a random variable with real values for the manually selected regions of interest were then calculated.*

The easiest parameter, and one that provides the most information about the reflectance of the analyzed blood samples is the average intensity of gray shades in the analyzed image. This parameter is calculated by summing the intensity of each pixel in the image and then by dividing the sum to the number of pixels. The sum obtained is called the Integrated Density of the Image and represents the second parameter analyzed in this paper. Because the investigated blood sample is a fluid in which bodies of different sizes, weights, and shapes float, I decided that a careful analysis of the distribution of the intensities of the gray shades reflected by it could generate information about the hazard degree in that sample. Thus, the parameters called ***skewness*** and ***kurtosis*** were analyzed.

4.4.5. STATISTICAL ANALYSIS OF THE DATA OBTAINED

Many values obtained by OCT continuing recording of whole blood coagulation must be statistically processed in order to define certain characteristics of the biological investigated processes and also the way they evolve over time. Microsoft Excel (Microsoft Corp., Redmond, WA, USA), together with the XLSTAT 2014 add-on for MS Excel (Addinsoft SARL, Paris, France) and IBM SPSS Statistics 20.0 (IBM Corporation, Armonk, NY, USA) were used for data analysis. The data obtained were recorded in Microsoft Excel files, then statistically processed in order to analyze the relationships between clinical and paraclinical data of patients.

CHAPTER 5 – RESULTS

5.1. Characteristics of the studied groups.

In this subchapter I have statistically highlighted the characteristics of the evaluated group in terms of gender, age, lifestyle-sedentarism and weight status (21 subjects - 26.58% having the weight above the limit for their gender and age, 98.74% of whom being a part of the patients with stroke. Many of the overweight patients are part of the stroke-diabetes group, 15 patients representing 55.56% of the population of this group). We observed that the number of sedentaries was higher than the number of active persons, representing 51 subjects (64.56%) of the total number of those studied, most of them, 30 (88.24%) being in the stroke group.

5.2. Results obtained at OCT evaluation.

Laser penetration – I have analyzed by hand the degree of penetration (Figure 5.7) and I noticed a significant difference between the two groups ($p < 0.01$). Thus, even if in absolute values the difference was only between 5 and 10 μm, the statistical analysis considered it to be highly significant.

5.2.1. Discontinuous analysis of blood samples

1. Average intensity of gray shades. The initial analysis of gray intensities revealed a different mean value in the two groups analyzed ($p < 0.001$), with a progressive decrease in the control group and an increase in the group including stroke patients (Figure 5.8).

2. Integrated density of gray shades. In order to have a global picture of cellular and molecular processes in the analyzed blood samples, the gray values of each pixel were summed in order to obtain the integrated density of the analyzed volume (Figure 5.9).

Immediately after the addition of calcium chloride, the integrated density of the two batches showed no differences between samples ($p = 0.6383$). Four minutes after the initiation of the reaction, there was an increase of this value of about two times in stroke patients, whereas in the control group

this value remained constant throughout the experiment. The difference between batches remains highly significant, with p values <0.001.

Taking into account that a huge amount of data has been analyzed, I have decided to look at how this data was distributed. Thus, the skewness and kurtosis of the gray values in each acquired image, over 300 images, were analyzed.

Skewness. The skewness analysis revealed an asymmetric distribution of the blood sample reflectivity in the control group, while the stroke group recorded relatively symmetrical distributions (Figure 5.10). Both groups showed positive values with a left deviation of the gray intensity values. This distribution shows that, at the level of normal blood volume, coagulation is not done in a random way, with the predominance of a particular process, making the recorded values to beasymmetric. This phenomenon does not seem to persist in patients with stroke, where the relatively symmetrical distribution of gray values masks a relatively symmetrical coagulation process due to the clotting points that randomly appeared in the analyzed volume.

Kurtosis. The kurtosis of the gray values in the analyzed sample identifies a distribution over a wide range of values in the control group compared to the group of stroke patients, the same random coagulation process was identified in stroke patients compared to normal, where there seems to be a predilection to certain processes (Figure 5.11).

The statistical analysis of these values revealed important differences between the two groups with p values well below the significance limit, starting from 0.1x10-6. After a detailed analysis of the way in which the gray values are distributed in the analyzed images, the existence of a possible dynamic and targeted process of establishing cell and molecular bonding in the blood sample coagulation process was found.

5.2.2 Continuous analysis of blood samples

Because there were indirect data of a targeted molecular and cellular process during normal coagulation, we decided to dynamically track this process by continuously investigating the coagulation for 15 minutes from its initiation. The analyzed parameters were kept the same as in the case of the discontinuous analysis, represented by the average gray intensity, the integrated density of these intensities, their skewness and kurtosis, the only major difference was that in case of continuous analysis the regression slope of the set of data recorded were analyzed in order to capture the variation in time of the analyzed parameters.

1. The mean intensity of gray shades recorded in stroke patients and in patients included in the control group, reveals a significant difference in tissue reflexivity, and there is a clear tendency for patients in the control group to show lower values but with a steep slope higher than in control group (Figure 5.12).

2. Integrated Density. The difference previously observed becomes even more evident (p <0.0001) when calculating the regression slope of the integrated density of these gray values in the analyzed sample (Figure 5.13), showing that the optical coherence technique can differentiate previously unidentifiable changes.

Data distribution appears to be much more random in ischemic stroke patients compared to patients included in the control group, analyzing skewness values, but both tend to initiate less random clotting processes. Although not very obviously statistically, the regression slopes of the two sets of data are different, showing that although there is a proportional coagulation tendency in the two groups, the times by which the two processes will end are different (p <0.0001) (Figure 5.14). By analyzing in detail the distribution of pixel intensity, I noticed that it is more homogeneous in healthy subjects compared to those suffering from ischemic stroke, thus revealing an inherently coagulation process, apparently altered in patients with ischemic stroke (p <0.0001) (Figure 5.15).

Linear regression slopes - the skewness of gray values in the two groups reveals a difference between the way coagulation is recorded through the OCT (p <0.0001).

Linear regression slopes - the kurtosis of the gray values in the two groups reveals a difference between how OCT coagulation is recorded ($p < 0.0001$).

These changes, though extremely encouraging, require a much more careful analysis to confirm the current data.

CONCLUSIONS

1. The aim of our study was to dynamically assess the changes in blood optic properties that occur during coagulation in a group of patients with ischemic stroke compared to a group of clinical and anamnestic healthy subjects.
2. A total number of 79 subjects, with an average age of 69.67 ± 7.77, of whom 41 (51.90%) men and 38 (48.10%) women, were examined by using optical coherence tomography - OCT and by using various clinical and biological investigations, they divided into three study groups: a group with ischemic stroke, a group with ischemic stroke and diabetes and a control group.
3. Analyzing the optical parameters in the subjects included in the study we found:
    - the degree of penetrance (distance) of the light beam through the coagulating blood was 5-10 μm lower in the ischemic stroke group compared to the control group, with a statistically significant difference ($p < 0.01$);
    - the mean intensity of the gray shades revealed a different mean value in the two groups analyzed ($p < 0.001$), with a progressive decrease in the control group and an increase in the ischemic stroke group;
    - the integrated density of the gray showed no differences between groups ($p = 0.6383$) for the baseline but it increased about two times after 4 minutes in stroke patients compared to control ($p < 0.00$);
    - skewness analysis revealed an asymmetric distribution of blood sample reflexes in the control group, while the ischemic stroke group showed relatively symmetric distributions, suggesting a random coagulation process;
    - kurtosis of gray values in the analyzed sample identified a distribution over a wide range of values in the control group compared to the group of patients with ischemic stroke, the same random coagulation process being identified in patients with ischemic stroke;
    - tissue reflectivity varied with time, and there was a clear tendency for stroke patients to show lower values but with a much higher slope compared to controls;
    - the regression slope of the integrated density of the gray values indicated a change in intensity during the coagulation period, pointing that the optical coherence technique may differentiate previously unidentifiable changes;
    - statistically, the regression slopes of the two sets of data were different, showing that although there was a proportional coagulation tendency in the two batches, the times by which the two processes will end are different ($p < 0.0001$);
    - pixel intensity distribution was more homogeneously present in healthy subjects compared to patients with ischemic stroke, thus highlighting that the intrinsic coagulation process apparently changed in stroke patients ($p < 0.0001$).
4. Analysis of classic haemostasis parameters:
    - did not show changes in the values for APTT, TQ, INR, percentage of prothrombin, parameters falling within the reference limits in all patients included in the study;
    - the concentration of fibrinogen was increased in the ischemic stroke group - diabetes (mean 416.66 ± 33.46 mg / dl), with 21.74% as compared to the control group ($p < 0.01$);

- platelet counts were significantly higher in patients with ischemic stroke than the control group (94.32% increase), although they remain within the reference limits (p = 0.000);

5. Patients with ischemic stroke had a cholesterol level (mean value of 236.42 mg / dl) higher with 27.76% than the control group.
6. The ability to identify intrinsic altered processes during coagulation in patients with ischemic stroke compared to the normal group through the OCT demonstrates the strength of this technique for analyzing soft or liquid tissues such as blood.

Final conclusion.

The study conducted in the present paper on the optical properties of fibrin clotted explored by OCT in patients with ischemic stroke is not mentioned in the literature.

From our results, the OCT seems to offer the possibility of identifying intrinsic altered processes during blood coagulation in patients with ischemic stroke compared to control group.

These changes, though extremely encouraging, require much deeper analysis in order to confirm the current data.

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