UNIVERSITY OF MEDICINE AND PHARMACY
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PhD THESIS

-ABSTRACT-

HISTOLOGICAL, MORPHOMETRICAL,
IMMUNOHISTOCHEMICAL, EXPERIMENTAL STUDY
ON MENINGOCEREBRAL VESSELS CHANGES
INDUCED BY RICH FATTY SATURATED ACIDS DIET

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KEY WORDS:
Atherosclerosis, Blood-brain barrier, High fat diet, Saturated fatty acids, Unsaturated fatty acids.

BACKGROUND

Even if stroke is a major cause of death in great countries of East Asia, as China an Japan, in Western Europe is „only" in third rank, i.e. 1.5 milions deceses every year (Wolinsky et al., 2009).

In the United States, mortality data from 2007 indicate that stroke accounted for 1 of every 18 deaths, vs. 1 of every 6 deaths from coronary heart disease [Roger et al., 2011]. Stroke mortality decreased over time, from 104.0 deaths/100,000 population in 1950 to 44.1 in 2008. This decreasing trend still maintains, ranked 4 in top 15 leading causes of death in 2010, after diseases of heart, malignant neoplasms and chronic lower respiratory diseases respectively, similar to European Union [Kunst et al., 2011].

In Eastern Europe, including Romania, stroke mortality dynamics was obvious different to Western and Central Europe. The rate was significantly greater in 1990-1996, followed by a light retrieval [Dolea et al., 2002]. Yet in 2005 outmatched coronary heart disease (30635 cases vs. 26633, from 123640 deceses) [Allender et al., 2008].

The statistical office of the European Union Eurostat data from 2001-2009 reveal that, in Romania, mortality by cerebrovascular diseases equals mortality by coronary heart disease in top, while in Western and Central Europe are in decline [European Commission, Eurostat, 2011]. Interestingly, there is a lack of correlation with mean total blood cholesterol decline in Romania from 5.4 mmol/l in '80s to less than 5 mmol/l în 2004-2008 [World Health Organization, 2011].

Our aim was to perform an experimental study in order to assess a correlation between fatty diet and meningocerebral arteries changes and, consequently, to analyse how fatty diet quality (unsaturated vs. saturated fatty acids) influences their wall structure.

CHAPTER I
MACRO- AND MICROVASCULARISATION OF CENTRAL NERVOUS SYSTEM, PHYSIOLOGY

The arterial blood supply to the brain is derived from two pair of arteries: the vertebral arteries and the internal carotid arteries. The paired vertebral arteries unite at the midline to form the basilar artery, which it bifurcates to form the paired posterior cerebral arteries. Through theirs branches, the vertebral and basilar arteries supply the medulla, pons, cerebellum, midbrain, caudal diencephalon, the occipital lobe and the inferior posterior temporal lobe.

The internal carotid arteries divide into two terminal branches: the anterior cerebral artery, which supplies the inferior and medial aspect of the frontal lobe and medial aspect of the parietal lobe, and the middle cerebral artery, which supplies the lateral portions of the orbital gyri and the frontal, parietal, and temporal lobes. Subpial branches of the middle cerebral arteries anastomose on the lateral surface of the cerebrum with subpial branches of the anterior and posterior cerebral arteries.

A major anastomotic connection between those two systems is the cerebral arterial circle (polygon of Willis). It consists of three major paired arteries: anterior, middle and posterior cerebral arteries, a pair of posterior communicating arteries, and a single anterior communicating artery (Crossman, 2005; Noback et al., 2005).

HISTOLOGY OF CEREBRAL ARTERIES

Cerebral arteries have different features from other arteries: their wall is thinner and external elastic lamina is absent. Instead, astrocytic processes, în parenchimal zone, and perivascular reticular sheath consisting of arachnoid trabeculae, in subpial zone, are present (Afifi, 2005).

Structurally, cerebral capillaries are similar to other capillaries, but they are surrounded by astrocytic processes, together integrating in blood-brain barrier. The blood–brain barrier is formed by brain endothelial cells, pericytes and astrocytes. Toghether with the neurons, they form the neurovascular unit (Hawkins and Davis, 2005).

PHISIOLOGY OF CEREBRAL PERFUSION

The blood–brain barrier protects the neurons from fluctuations in plasma composition that is it plays an important role in the homeostasis of the neuron microenvironment. Its specificity consists of complex tight junctions, and transport systems which regulate molecular traffic (Abbot, 2002).

The lack of classic capillary recruitment, due to the thinner media layer of arterioles, is supplied by the presence of functional recruitment. The most relevant physiologic influencers are: diffusibility of oxygen and glucose, capillary heterogeneity, coupling between in cerebral bood flow and cerebral metabolic rate for glucose (Paulson et al., 2010).

CHAPTER II
CEREBRAL ATHEROSCLEROSIS

The current classification as proposed by the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association, comprise six types, in fact stages in atheroma evolution. Type I (initial lesion) and type II (progression-prone = type IIa, progression-resistant = type IIb) correspond to early lesion, i.e. fatty dot or streak. Type III is intermediate lesion (preatheroma). Type IV represents fibrous cap atheroma, and type V atheroma with 3 features: Va (fibroatheroma), Vb (calcified plaque), and Vc fibrous plaque). Type VI lesion includes lesions with surface defect, hematoma, hemmorrhage and/or thrombotic deposit, namely complicated lesion or plaque (Stary et al., 1994, 1995). Instead, Virmani et al. (2000) propose a a simplified classification that relies on descriptive morphology: nonatherosclerotic intimal lesions (intimal thickening and intimal xanthoma, or "fatty streak") progressive atherosclerotic lesions (pathological intimal thickening, fibrous cap atheroma, thin fibrous cap atheroma with plaque rupture, calcified nodule, and fibrocalcific plaque).

CHAPTER III
DIETARY LIPIDS AND SATURATED FATTY ACIDS

Fatty acids play key roles in metabolism: effective source of energy (high, low weight/energy unit), transport form of energy as blood lipids (triacylglycerol in lipoproteins), storage of energy (adipose tissue), component of cell membranes (phospholipids), thermal and electrical insulating, mechanical protection, and signals for gene regulation and transcription (eicosanoids). Free fatty acids and their salts may function as detergents to initiate the formation of micelles due to their amphiphatic properties (Rustan A., Drevon C, 2001).

The mechanisms by which high fatty diet could affect brain consist in a modified lipid profile which disturbs the metabolic parameters and reduces brain nitric oxide resulting in brain lipid peroxidation (Amin et al., 2011). Atherogenic liproteins, as apolipoprotein B, initiate the process of atherogenesis. These retained lipoproteins induce an inflammatory response dominated by macrophages an T-cells (Tabas et al., 2007).

CHAPTER IV
EXPERIMENTAL STUDY

All experiments were performed in accordance with the council directive (86/609/EEC) of European communities, after Ethics Committee of University of Medicine and Pharmacy of Craiova approval. (No. 12/9.03.2012)

72 adult Wistar rats, weighing between 200 and 300 g were randomly divided into 7 groups. 1 group consists in 18 Wistar rats fed with standard diet, 6 experimental groups of 9 Wistar rats each fed with fatty diet as follows: 6 rats of each group fed with rich saturated fatty acids diet (palm oil) and 3 rats fed with rich unsaturated fatty acids diet (untar).

Diet and water were provided ad libitum. The animals were housed in cages on clean paddy husk beddings and were maintained under controlled temperature of 21° ± 3°C, humidity ratio 45-65%, with a normal 12-hour light/12 hour dark cycle. Animal ethical guidelines were followed throughout the experimental period.

Animals from each group were sacrificed after 12, 18, 24, 30, 36 and, respectively one year.

Animals were sacrificed (in accordance with Bioethics Committee) by decapitation, after anesthesia induced with Narcoxyl 0.1mg/g body weight and Ketamine 0.3mg/g body weight hypodermic injection.

The whole brain was removed and fixed by immersion at room temperature in 10% buffered formalin for 24 h. Then, the brains were cut first mediosagitally, then left hemisphere was cut coronally at 3 mm intervals and the right one parasagitally at the same 3 mm intervals. Paraffin-embedded tissue 3-5 μm thickness sections were stained with hematoxylin-eosin (for basic histological examination), and Masson's trichrome stain for distinguishing cells from surrounding connective tissue. Unstained sections were moved onto polilysine microscope slides, and then processed with Anti-alpha smooth muscle Actin antibody for immunohistochemical study of arteriolar media layer.

The tissue samples were then examined under Nikon Eclipse 55i (Nikon, Apidrag, Romania) light microscope. Images are digitized and captured with 5 Megapixels CCD cooling camera connected to a computer with Image Proplus 7 AMS software (Media Cybernetics. Inc. Buckinghamshire, UK).

CHAPTER V
HISTOLOGICAL STUDY

First time point was after 12 weeks, then after 18 weeks from the begining of experiment.

In the first group (rich saturated fatty acids diet), basic histological examination revealed that earlest lesions appeared on subpial vessels. Occasionally, we observed minute subpial hemorrhage, extravessel red cells sparing subarachnoid space. Also, at a more accurate examination, we emphasized minute vacuoles, located into intima and media layers of superficial (pial) artery wall. These vacuoles, usually large and unique, unevenly spread out circumferentially, outside internal elastic lamina. The presence of these microscopic lesions denoted the appearance of lipid accumulation in blood vessels wall, especially arterioles, both in tunica intima as in tunica media. We considered that rich saturated fatty acids diet determined the lipid crossing, especially saturated fatty acids, through endothelium in intimal and subintimal layers, to smooth muscle cells of the arteriolar media.

Minute progressive lesions was seen also into cerebral parenchyma. Due to modicum arteriolar wall thickness, media beeing almost absent, intraparietal lesions were detected with difficulty. Instead, we observed sometimes early intraluminal thrombosis, and perivascular edema, shaped as a free optical muff. This perivascular edema spread along branches to capillaries.

In our study, we often found perivascular edema, arround arterioles, venules and capillaries. We considered this is not an artifact, because its bi- and tridimensional distribution was uneven, such changes neighbouring with arterioles without surrounding edema. This aspect denoted the presence of some microscopically lesions in blood-brain barrier, presumably at endothelial junctions. We believed that perivascular edema had a vasogenous origin, due to biochemical changes occurred especially in intima.

In rats sacrificated at 24 and 30 weeks after the onset of experiment, more conspicuous lesions were seen, with both circumferentially and deeply wall spread, and its obviously thickening.

Some of rats subjected to a rich saturated fatty acids diet showed the three lesions previously founded, i.e. vacuolation, thrombosis, and perivascular edema, but more pronounced. Intraparenchymal arteries had agglutinated or lysed red cells, sometimes with parietal thrombosis tendency. Vacuoles were found in all arteriolar wall layers (the intima, the media, and the adventitia), with an almost evenly circumferentially scattering. The complete perivascular circumferential edema disconnected arterioles even from theirs satellite venules.

The relationship between arteriolar wall structural changes and hemorrhage was intricate. Hemorrhage could be related to a thickening pial arteriolar wall and a consecutive vacuolation with or without its embrittlement. Thereby, a meningeal diffuse hemorrhage occured. This time were not only some red blood cells in subpial zone, but a massive subarachnoid hemorrhage. As arteriole was detached from surrounding parenchyma by numerous red blood cells extravasated, we considered that the hemorrhage's magnitude, primary subpial, determined pia mater lesion, and successive flooding in subarachnoid space.

In rats subjected to a rich unsaturated fatty acids diet, lesions seemed to evolve rather to a fibrous pattern than a dilaceration with successive hemorrhage. Even if the subintimal arteriolar vacuolation was present, it did not dilacerate collagen fibres; on the contrary, an intramural fibrosis occured. Even if a lesional polimophism was observed, intramural vacuolations were accompanied with adventital fibrosis, highlighted by Masson's trichrome stain.

CHAPTER VI
IMMUNOHISTOCHEMICAL STUDY

Immunohistochemical examinations were performed only on animals sacrificed at the end of experiment, after 48 weeks. In rats subjected to a rich unsaturated fatty acids diet, alfa-SMA markers pointed out complete luminal thrombosis, followed by an organized thrombus with multiple capillary channels padded with endothelial cells. It is not a „restutio ad integro", because tunica media was scanty immunostained with antibodies to alpha-smooth muscle actin. Thus, vessels newly framed in organized thrombus allowed to resume the blood flow, but this flow is difficult due to its increased parietal friction and lack of dynamic regulation.

CHAPTER VII
MORPHOMETRICAL STUDY

Morphometry on large arteries, especially carotid intima-media thickness, are currently widely used in randomized controlled trials using ultrasound techniques(Bots et al., 2003; Finn et al 2010). Instead, small cerebral arteries alterations due to aging are less known. Images acquired were processed with Lucia Net software for morphometry. For each artery, we identified and the software measured: di = minimum inner (luminal) diameter; Di = maximum inner (luminal) diameter; do = minimum outer diameter; Do = maximum outer diameter; tw = minimum wall thickness; Tw = maximum wall thickness; Ai = inner (luminal) area of arterial section; Ao = outer (total) area of arterial section.

These measured data were used to calculate some indexes: Thickness uniformity index: Ti = tw / Tw; Wall section area: Aw = Ao – Ai; Thickness area ratio: Tr = Aw / Ao.

Average and standard deviation were calculated for all arguments and indexes, respecting groups and time points.

Hematoxylin-eosin slides were more appropriate for morphometrical analysis than slides with silver salt stain. Uneven feature of reticular fiber, especially in atherosclerotic arteries, makes inner and outer wall boundaries difficult to be identified.

Another challenge was due to random sections in account with tract of near-hippocampus arteries. Therefore, the number of arteries fit to morphometrical analysis, i.e. cross-sectioned, was different in many cases.

Neither morphometrical analysis, nor statistical analysis shows suggestive change related to diet or time points. Even when average seems to change significantly, standard deviation is too large to be taken into account. Thickness uniformity index varies from 0.34 ± 0.03 to 0.29 ± 0.03, in normal diet and 0.34 ± 0.05 to 0.30 ± 0.04, in fatty diet. More useful is those related to areas: thickness area ratio rise from 0.77 ± 0.01 after 3 months of normal diet to 0.82 ± 0.02 after 5 months. Group subjected to fatty diet had the same tendency, even little more: from 0.77 ± 0.02 after 3 months to 0.84 ± 0.01 after 5 months. Ratio between wall arterial cross-section area and luminal area is the most suggestive argument to evaluate structural changes in cerebral arteries evolution under normal and pathological conditions.

Our experimental study is concordant with imaging human studies (O'Leary et al., 2010) concerning "natural" arterial evolution to thicken its wall. More than structural changes themselves, impact on endothelial permeability is worrying. Instead, effects of fatty diet on cerebral arterial wall thickness are not so manifest.

Fatty diet affects arterial wall structure in a vaguish manner comparatively to arterial ageing.

CONCLUSIONS

1. A rich saturated fatty acids diet determined the appearance of vacuoles, presumably lipids, in arteriolar wall, mostly subintimal, associated with thrombosis, microhemorrhage and perivascular edema.

2. In the first stages of our experiment, vascular meningocerebral changes were modicum in intensity.

3. The magnitude of atheromatous process rose in ultimate stage of experiment, due to the quantitative growth of saturated fatty acids in diet.

4. Rich saturated fatty acids diet produced widespread lesions than rich unsaturated fatty acids diet.

SELECTED BIBLIOGRAPHY:

1. **Abbott J**. *Astrocyte–endothelial interactions and blood–brain barrier permeability.* J Anat. 2002 June; 200(6): 629–638.
2. **Afifi A., Bergman R.** *Functional Neuroanatomy: Text and Atlas*, 2nd Edition. McGraw-Hill, 2005: 348-354
3. **Allender S., Scarborough P., Peto V., Rayner M., Leal J., Luengo-Fernandez R., Gray .** *European cardiovascular disease statistics 2008*, University of Oxford, 2008.
4. **Amin K., Kamel H., Abd Eltawab M**. *The relation of high fat diet, metabolic disturbances and brain oxidative dysfunction: modulation by hydroxy citric acid*. Lipids in Health and Disease, 2011, 10:74-84
5. **Bots M, Evans G, Riley W, Grobbee D.** *Carotid Intima-Media Thickness Measurements in Intervention Studies*. Stroke, 34: 2985-2994, 2003.
6. **Crossman A.R.,** *Vascular supply of the brain.* Gray's Anatomy The Thirthy-Ninth Edition, Susan Standring editor in chief, Elsevire Edition, 2005, pp 295-303
7. **Dolea C., Nolte E., McKee M.** *Changing life expectancy in Romania after the transition.* J Epidemiol Community Health 2002;56:444–449.

8. **European Commission, Eurostat. Health statistics. 2011**. © European Union, 2011 ISBN: 978-92-79-18757-5.
9. **Finn AV, Kolodgie FD, Virmani R.** *Correlation between carotid intimal/medial thickness and atherosclerosis: a point of view from pathology.* Arterioscler Thromb Vasc Biol, 30:177-81, 2010.
10. **Hawkins B.T., Davis T.P.** *The Blood-Brain Barrier/Neurovascular Unit in Health and Disease.* Pharmacological Reviews June 2005 vol. 57 no. 2 173-185
11. **Kunst AE, Amiri M, Janssen F.** *The decline in stroke mortality: exploration of future trends in 7 Western European countries.* Stroke. 2011,42(8):2126-30.
12. **Noback C, Strominger N, Ruggiero D, Demarest R.** *The human nervous system: structure and function*. 6th ed. Humana Press Inc. 2005: 77-88.
13. **O'Leary D, Bots M**. *Imaging of atherosclerosis: carotid intima–media thickness*. Eur Heart J, 31: 1682-1689, 2010.
14. **Paulson OB, Hasselbalch SG, Rostrup E, Moos Knudsen G, Pelligrino D**. *Cerebral blood flow response to functional activation*. Journal of Cerebral Blood Flow & Metabolism (2010) 30, 2–14
15. **Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, Carnethon MR, Dai S, de Simone G, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Greenlund KJ, Hailpern SM, Heit JA, Ho PM, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, McDermott MM, Meigs JB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Rosamond WD, Sorlie PD, Stafford RS, Turan TN, Turner MB, Wong ND, Wylie-Rosett J;** on behalf of the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Heart disease and stroke statistics—2011 update: a report from the American Heart Association*. Circulation. 2011;123:e18–e209
16. **Rustan A., Drevon C**. *Fatty Acids: Structures and Properties. ENCYCLOPEDIA OF LIFE SCIENCES* / & 2001 Nature Publishing Group :1-7.
17. **Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W Jr, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW.** *A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis,* American Heart Association. Arterioscler Thromb Vasc Biol. Sep 1995;15(9):1512-31
18. **Stary HC, Chandler B, Glagov S, Guyton JR, Insull W Jr, Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD, Wissler RW**. *A definition of initial, fatty streak, and intermediate lesions of atherosclerosis: a report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association.* Circulation 1994; 89:2462-2478.
19. **Tabas I, Williams KJ, Borén J.** Subendothelial lipoprotein retention as the initiating process in atherosclerosis. Update and therapeutic implications. Circulation 2007;116:1832-1844.
20. **Virmani R., Kolodgie F., Burke A., Farb A., Schwartz S.** *Lessons From Sudden Coronary Death : A Comprehensive Morphological Classification Scheme for Atherosclerotic Lesions.* Arterioscler Thromb Vasc Biol 2000, 20:1262-1275.
21. **Wolinsky F, Suzanne Bentler, Elizabeth Cook, Elizabeth Chrischilles, Li Liu, Kara Wright, Geweke J, Obrizan M, Claire Pavlik, Ohsfeldt R, Jones M, Wallace R, Rosenthal G.** *A 12-year prospective study of stroke risk in older Medicare beneficiaries.* BMC Geriatrics 2009, 9:17.
22. **World Health Organization** - NCD Country Profiles, Romania 2011