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Cerebral microvascular changes induced by rich cholesterol and saturated fatty acid diet in Wistar rats

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Abstract

The impact of an excess of fatty acids in the diet on cardiovascular diseases has been studied and discussed both in human and animal studies. Generally, excessive saturated fats increase the risk, while unsaturated fats are considered less harmful. Our aim was to perform an experimental study in order to analyze how fatty diet quality (unsaturated *vs.* saturated fatty acids) influences atherogenesis. *Materials and Methods*: In our experimental study, 18 adult Wistar rats were randomly divided into two equal groups. One group was subjected to a rich unsaturated fatty acid diet (untar) and the other group to a rich saturated one (palm oil). Three animals from each group were sacrificed after 12, 18, and 48 weeks. The brain was removed and microscopically examined after Hematoxylin–Eosin, Orcein and Masson's trichrome classical staining, and after immunohistochemical marking using the anti-alpha smooth muscle actin antibody. *Results*: Rats sacrificed after 12 weeks revealed modicum lesions, as intimal vacuoles or minute intraluminal thrombosis, and cerebral parenchymal edema. After 18 weeks, some of rats subjected to a rich saturated fatty acid diet presented vacuoles found in all arteriolar wall layers, and a tendency towards parietal thrombosis. In rats subjected to a rich unsaturated fatty acid diet, the subintimal arteriolar vacuolization was associated with an intramural and adventitial fibrosis. In rats sacrificed after 48 weeks, lesional polymorphism was pronounced, but in rats subjected to a rich unsaturated fatty acid diet complete luminal thrombosis was followed by a an organized thrombus with multiple capillary channels. Although in Wistar rats atherosclerosis appeared only after intensive changes in diet, different experimental studies showed that, in transgenic rats, rich saturated fatty acid diet induced progressive atherosclerotic lesions, resembling those observed by us, but also some aspects described in human pathology. *Conclusions*: Our experimental study reveals differences in atherogenesis under saturated *vs.* unsaturated fatty diet.

Keywords: atherosclerosis, blood-brain barrier, high fat diet, saturated fatty acids, unsaturated fatty acids.

Introduction

Worldwide the mortality rate from cardiovascular diseases is still the leading cause of death: in 2008 there were 17 million cases (48% of deaths by chronic diseases) *vs.* cancer (7.6 million cases, *i.e.* 21%), and chronic obstructive pulmonary diseases (4.2 million cases, *i.e.* 12%). In Romania, cardiovascular diseases were in top but with a higher rate (59%). Among cardiovascular diseases, stroke was ranked second worldwide, with 6.15 million deaths (10.8%), ischemic heart diseases being in top. It is predicted that the top four leading causes of death in the world in 2030 will maintain the same ranks [1].

In the United States, mortality data from 2007 indicates that stroke accounted for one of every 18 deaths *vs.* one of every six deaths for coronary heart disease [2]. Stroke mortality decreased over time, from 104 deaths/100 000 population in 1950 to 44.1 in 2008 [3]. This decreasing trend still maintains, ranked four in the top 15 leading causes of death in 2010, after heart diseases, malignant neoplasms, and chronic lower respiratory diseases [4], similar to the European Union [5, 6].

In Eastern Europe, including Romania, stroke mortality dynamics was different from Western and Central Europe [7, 8] in an obvious manner. The rate was significantly greater in 1990–1996, followed by a slight decrease [9]. Yet, in 2005, it outmatched the coronary heart disease (30 635 cases *vs.* 26 633, of 123 640 deaths [10].

The *Statistical Office of the European Union* (*EUROSTAT*) data from 2001–2009 revealed that, in Romania, the mortality by cerebrovascular diseases equals the mortality by coronary heart disease, while in Western and Central Europe they are declining [11]. Interestingly, there is a lack of correlation with the mean total blood cholesterol decline in Romania, from 5.4 mmol/L in the '80s, to less than 5 mmol/L between 2004 and 2008 [12].

In general, rich saturated fatty acid and cholesterol diet increase the risk for the development of obesity, diabetes and cardiovascular diseases, while rich unsaturated fatty acids are considered less harmful [13].

Our aim was to perform an experimental study in order to assess a correlation between fatty diet and changes of the meningocerebral arteries and,

consequently, to analyze how fatty diet type (unsaturated *vs.* saturated fatty acids) influences their wall structure.

Materials and Methods

All experiments were performed following the approval from the Ethics Committee of the University of Medicine and Pharmacy of Craiova, Romania, in accordance with the European Council Directive No. 86/609/EEC.

Eighteen adult Wistar rats, weighing between 200 and 300 g, were randomly divided into two groups of nine animals each. The first group was subjected to a rich unsaturated fatty acid diet, and the second group was subjected to a rich saturated fatty acid diet.

Rich unsaturated fatty acid diet (untar, *i.e.* margarine-butter blend 70/30 percents) per 100 g: energy 714 kcal; water 17.07 g; proteins 0.31 g; carbohydrates 0.77 g; total lipids (fat) 80.32 g, saturated fatty acids 14.19 g, mono-unsaturated fatty acids 30.29 g, polyunsaturated fatty acids 24.17 g, cholesterol 12 g. Rich saturated fatty acid diet (palm oil) per 100 g: energy 884 kcal; water, proteins, and carbohydrates 0 g; total lipids (fat) 100 g, saturated fatty acids 49.3 g, monounsaturated fatty acids 37 g, polyunsaturated fatty acids 9.3 g, cholesterol 0 g. Thus, saturated fatty acids are on a par with unsaturated fatty acids [14].

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 ± 3^0C, humidity ratio 45–65%, with a normal 12 hours light/ 12 hours dark cycle. Animal ethical guidelines were followed throughout the experimental period.

Three animals from each group were sacrificed at three time points, after 12, 18, and 48 weeks respectively. Animals were sacrificed (in accordance with Bioethics Committee) by decapitation, after anesthesia induced by hypodermic injection with Narcoxyl 0.1 mg/g body weight and Ketamine 0.3 mg/g body weight. The whole brain was removed and fixed by immersion at room temperature in 10% buffered formalin for 24 hours. Then, the brains were cut first mediosagitally, and then the left hemisphere was cut coronally at 3 mm intervals and the right one parasagitally, also at 3 mm intervals. The biologic material was embedded in paraffin and 3–5 μm-thick sections were cut, which were stained with Hematoxylin–Eosin (for basic histological examination), Orcein (for elastic tissue), and Masson's trichrome stain for distinguishing cells from surrounding connective tissue. Other unstained sections were moved onto poly-L-Lysine microscope slides, and then processed with anti-α-smooth muscle actin (α-SMA) antibody for immuno-histochemical study of arteriolar medial layer. Tissue samples were then examined using a Nikon Eclipse 55i light microscope (Nikon, Apidrag, Romania). Images were digitized and captured with a 5-Megapixel CCD cooled camera using the Image ProPlus 7 AMS software (Media Cybernetics, Inc., Buckinghamshire, UK).

Results

The first time point was at 12 weeks from the beginning of the experiment.

In the first group (rich unsaturated fatty acid diet), basic histological examination revealed that the earliest lesions appeared on subpial vessels. Occasionally, we observed minute subpial hemorrhages that spared the subarachnoid space. Also, at a more detailed examination, we emphasized minute vacuoles, located within the intimal and medial layers of the superficial (pial) artery wall. These vacuoles, usually large and unique, showed an uneven circumferential spreading pattern, outside the internal elastic lamina (Figure 1). High magnification photomicrographs of Orcein stained slides showed that these vacuoles partially affected the integrity of the internal elastic lamina. This aspect was seen only in pial artery wall, but not in *vasa vasorum* (Figure 2). The presence of these microscopic lesions denoted the development of lipid accumulations in the blood vessel wall, especially of arterioles, both within the intimal and medial layer. We considered that rich saturated fatty acid diet determined the passage of lipids, especially saturated fatty acids, through the endothelium, towards the intimal and subintimal layers, and smooth muscle cells of the arteriolar media.

Minute progressive lesions were also seen in the cerebral parenchyma. Due to the reduced arteriolar wall thickness, with an almost absent medial layer, intra-parietal lesions were detected with difficulty. Instead, we sometimes observed early intraluminal thrombosis, and perivascular edema, shaped as an optically empty sheath (Figure 3). This perivascular edema spread along the branches towards the capillaries.

In our study, we often found perivascular edema, around arterioles, venules and capillaries. We considered that this is not an artifact because of its heterogeneous bi- and three-dimensional distribution, such changes neighboring arterioles without surrounding edema. This aspect denoted the presence of some microscopic lesions of the blood-brain barrier, presumably involving the endothelial junctions. We believe that perivascular edema had a vasogenous origin, due to biochemical changes that occurred especially within the intima.

In rats sacrificed at 18 weeks after the onset of the experiment, more conspicuous lesions were seen, with both circumferential and deep extension within the obviously thickened wall.

Some of rats subjected to a rich saturated fatty acid diet showed the three lesions previously found (*i.e.* vacuolation, thrombosis, and perivascular edema), but more pronounced. Intraparenchymal arteries showed agglutinated or lysed red cells, sometimes with tendency towards parietal thrombosis. Vacuoles were found in all arteriolar wall layers (intima, media, and adventitia), with an almost even circumferential disposition. Complete perivascular circumferential edema disconnected arterioles even from theirs satellite venules (Figure 4).

The relationship between arteriolar wall structural changes and hemorrhage was intricate. Hemorrhage could be related to a thickened pial arteriolar wall and a consecutive vacuolation with or without its embrittlement. In the case of pial vessels, the consequence was the diffuse meningeal hemorrhage. However, this time it was not just some red blood cells in the subpial zone, but a massive subarachnoid hemorrhage. As the

arteriole was detached from the surrounding parenchyma by numerous extravasated red blood cells, we considered that the magnitude of the hemorrhage, primarily subpial, lead to the impairment of the pia mater, and consequent flooding of the subarachnoid space (Figure 5).

In rats subjected to a rich unsaturated fatty acid diet, lesions seemed to evolve rather towards a fibrous pattern than a dilaceration with successive hemorrhage. Even if the subintimal arteriolar vacuolation was present, it did not dilacerate collagen fibers; on the contrary, an intramural fibrosis occurred. Even if a lesional polymorphism was observed, intramural vacuolizations were accompanied by adventital fibrosis, highlighted by Masson's trichrome stain (Figure 6).

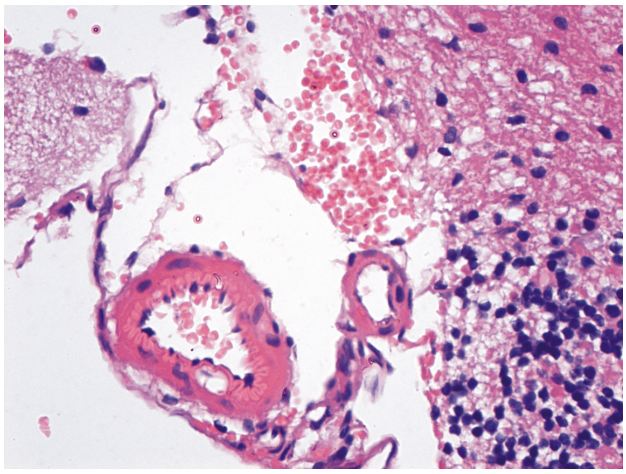

Figure 1 – ***Cerebral microhemorrhage and arteriolar vacuolization (HE stain, ×400).***

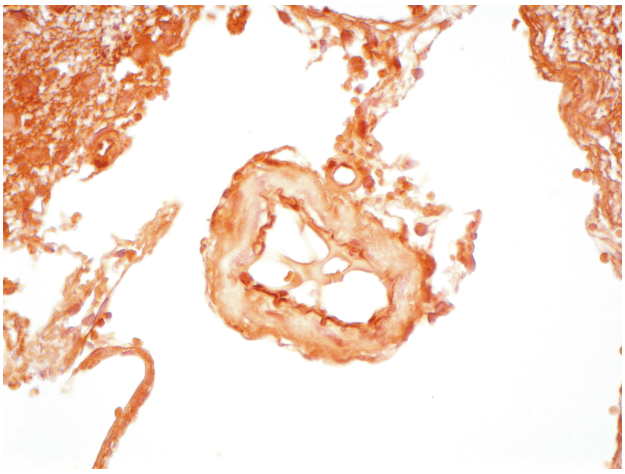

Figure 2 – ***Arteriolar vacuolization partially affecting the internal elastic lamina integrity (Orcein stain, ×400).***

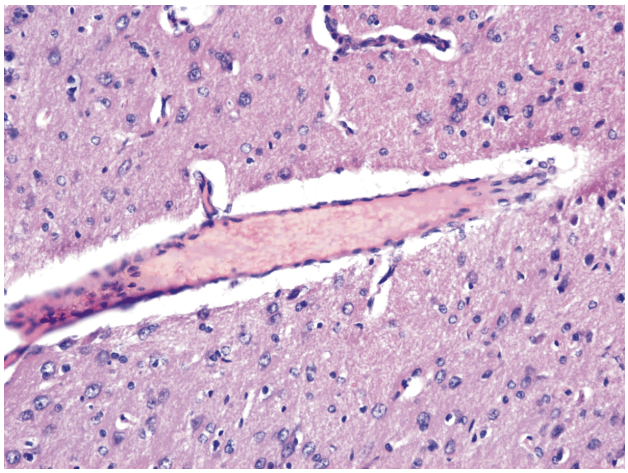

Figure 3 – ***Intraparenchymal perivascular edema (HE stain, ×400).***

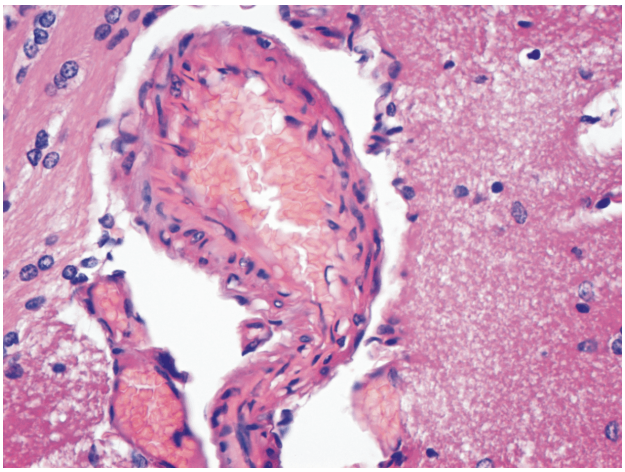

Figure 4 – ***Arteriolar occlusion by agglutinated and lysed red cells, with tendency towards parietal thrombosis (HE stain, ×400).***

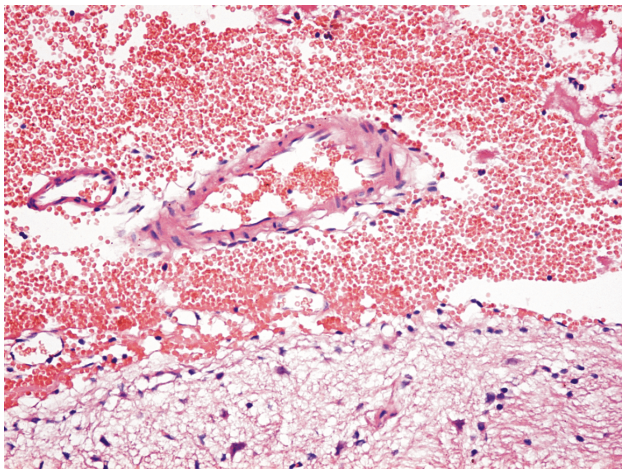

Figure 5 – ***Microhemorrhages with structural changes of the wall and embrittlement (HE stain, ×200).***

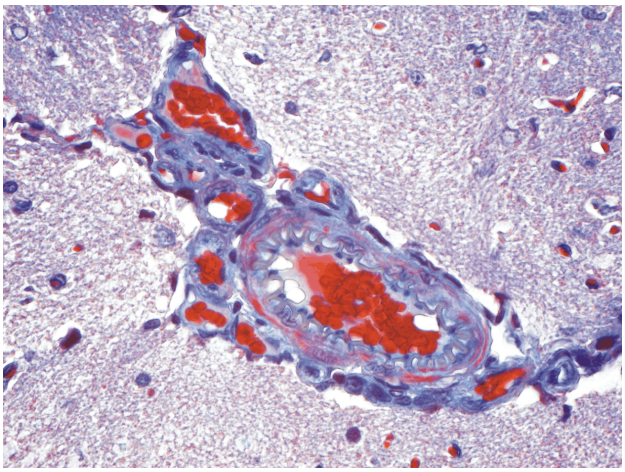

Figure 6 – ***Arterioles with numerous vacuoles, dissociated smooth muscle cells and adventitial fibrosis (Masson's trichrome stain, ×400).***

Immunohistochemical investigations were performed only on animals sacrificed at the end of the experiment, after 48 weeks. In rats subjected to a rich unsaturated fatty acid diet, α-SMA immunostaining highlighted the complete luminal thrombosis, followed by an organized thrombus with multiple capillary channels lined by endothelial cells. It is not a *restitutio ad integrum*, since the media showed weak immunostainig for anti-α-SMA antibody. Thus, neoformation vessels within the organized thrombus allow, at least in theory, the repermeabilization of the fully thrombosed artery. However, this blood flow is impaired due to the increased parietal friction and lack of dynamic regulation (Figure 7).

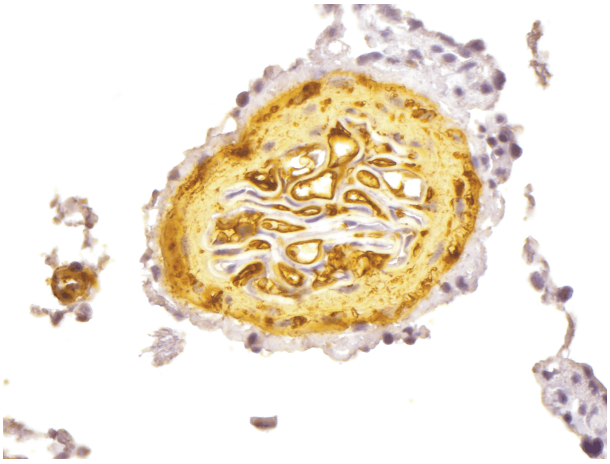

Figure 7 – ***Arteriolar lumen filled with an organized thrombus with multiple capillary channels (α-SMA immunohistochemistry, ×400).***

Discussion

First, we have to take into account the animal models used. Wistar rats, although extensively used in experimental studies, are naturally highly resistant to diet-induced atherosclerosis [15]. Appropriate animal models for atherosclerosis are pigs. They develop atherosclerosis even after normal diet [16, 17]. However, the maintenance of pigs is expensive and difficult, beyond the capabilities of common laboratories [18]. Instead, more appropriate experimental animals for research on atherosclerosis are mini pigs [19].

In rats, the atherosclerotic process develops only after intensive changes in diet [20]. Transgenic rat models develop lesions resembling atherosclerosis, either for human cholesteryl ester transfer protein [21], or insulin resistance [22].

Following comparison of our results to published data, we found that lesions in rat models rarely progress beyond the stage of atheroma. This evolution was often irregular, situation quite atypical compared to the human disease [23, 24].

Frequently, within the same group, and even in the same subject (rat), we noted wide variations ranging from incipient or absent lesion, to complicated lesion. The perivascular edema is the same to that induced by ischemia achieved through internal carotid artery ligation [25]. Lesional polymorphism is more pronounced especially in rats sacrificed at 18 weeks. This stage corresponds to the late phase in humans that is the phase when most frequent early signs of cerebral atherosclerosis occur.

Surprisingly, but in accordance with newly published data [26], the microscopic appearance cannot be correlated with the fatty diet, saturated or unsaturated. Morphometric studies are more suggestive for the evaluation of structural changes in cerebral arteries [27].

In rats sacrificed at 48 weeks, corresponding to the old age in men, atherosclerotic lesions were more prominent, but showed the same lesional polymorphism and increased variability, both within the same group and individual. The only suggestive aspect, also the most interesting one, was the repermeabilization of the thrombus by numerous neoformation capillaries. This aspect was seen only in rats subjected to rich unsaturated fatty acid diet and is rarely described in the literature. Virmani R *et al.* (2000) assumed that these types of lesions are the result of healed erosions [24]. Occasionally, such capillaries may derive from *vasa vasorum* [28], neointima being formed by arterial smooth muscle cells [29]. Even though we did not find α-SMA-positive smooth muscle cells, this mechanism is not excluded, because the phenotypes of smooth muscle expressed in atheroma is changing by "modulation" [30], including α-actin [31].

In the case of lesional polymorphisms, one cannot make a clear distinction between changes in smooth muscle cells from subjects in different experimental groups or individuals within the same group.

Vacuolar lesion topography, with assumed lipid storage, is intricate. Vacuoles may show subintimal, adventitial location, or within the smooth muscle layer (media). In this case, vacuoles, as well as fibrosis, can sometimes dissociate smooth muscle cells, affecting the continuity of the contractile structure, but maintaining the integrity of each cell. In other instances, smooth muscle cells are themselves submitted to a dystrophic process, eventually leading to complete lysis.

Such features explain damages in neurohumoral control of cerebral blood flow in microstroke [32], especially associated with arterial hypertension, embolism in atrial fibrillation, and diabetic angiopathy [33].

In healed thrombosis, the lack of smooth muscle cells around well endothelized neovessels excludes the possibility of *restitutio ad integrum*. In fact, there is a partial restitution of blood flow, with no local regulation. Thereby, minimal detectable changes may facilitate interpretations of patient-reported outcomes after stroke rehabilitation [34, 35], especially in the vulnerable post-hospital transition period [36].

Conclusions

The rich unsaturated fatty acid diet determined the appearance of vacuoles, presumably lipids, within the arteriolar wall, mostly subintimal, associated with thrombosis, microhemorrhage and perivascular edema. In the first stages of our experiment, vascular meningo-cerebral changes showed a moderate intensity. The magnitude of the atheromatous process rose in the last

stage of the experiment, because of an increased quantity of saturated fatty acids in the diet. When compared to the rich unsaturated fatty acid diet, the rich saturated fatty acid diet produced widespread lesions.

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Contribution Note

All authors have equally contributed to this work.

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