A Contribution to the Morphological Study of Acute Changes in the Blood Vessels of the Central Nervous System of Rats Following Irradiation with High-Energy (4 GeV/Nucleon) Helium Ions

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Abstract. Rats were irradiated with helium ions (4 GeV/nucleon; 2 or 4 Gy). After 4—9 hours or three days, a perfusion was performed and sections of the cerebral cortex were investigated under light and electron microscopes. Changes observed in the vessels of the telencephalic cortex are described. Most of the vessels in the sections analysed showed no signs of damage. In some cases there was a dilated perivascular space; a comparison of its occurrence in irradiated and control animals showed a statistically significant increase in frequency of the phenomenon at a shorter interval after irradiation with a larger dose. Electron microscopical analysis showed that the main locality of damage was the border of the perivascular foot of the astrocytes, which exhibited various degrees of edema. The discussion stresses the peculiarities of the interaction of helium ions with living tissues, particularly the heterogeneity of the distribution of absorbed energy. The consequence is in accordance with the nature of the changes observed.

Key words: Cerebral vessels ultrastructure — Edema of astrocytes — Irradiaton with high-energy helium ions — Perfusion fixation artifacts — Perivascular space

Introduction

The study of changes in the morphology and functional properties of blood vessels of the central nervous system is an important part of the research on damage to the nervous system caused by irradiation. The nature of these changes, their extent and consequences for the nerve tissue depend on dose and type of radiation, on fractionation and the volume of irradiated tissue, on the interval after irradiation, as well as on the type of the organism. In the present paper we describe morphological findings in the cerebral cortex of rats shortly after irradiation with high-energy helium ions. After relatively small doses (2 or 4 Gy) of radiation, the main observation was dilation of the perivascular space, the absence of which is

important for the maintaining of the blood-brain barrier (Hager 1961). The contribution of the dilation of the perivascular space to acute or delayed radionecrosis of nerve tissue is considered by some authors as having little if any significance (e.g. Caveness et al. 1968). On the other hand, Larsson (1960) has considered vascular damage as the basic pathogenetic principle in the development of delayed radionecrosis. It seems that important factors in this process are the radiation dose and the time elapsed after irradiation: following doses in the range of 100 Gy or more and at high dose rates, there is an immediate increase in vascular permeability, and acute death of nerve cells and glial elements occurs (Klatzo et al. 1961). Following a dose of 800 R gamma radiation, which is better comparable with doses used (2 or 4 Gy), Ostenda (1979) found astrocyte changes within three hours after irradiation; subsequently the prevalent changes included swelling and degeneration which progressed to irreversible destruction. Our report contains information on the effect of still smaller doses of "unconventional" radiation, i.e. high-energy helium ions; on interaction of these particles with matter, energy absorption is very heterogenous. The morphological findings were quantified and they are illustrated with photographs of largish areas taken from the light microscope. Selected sites were analysed using an electron microscope.

Materials and Methods

Experimental animals: Female Wistar rats weighing on average 180 g, aged two months.

Irradiation: Helium ions with an energy of 4 GeV/nucleon, produced in a synchrophasotron of the Joint Institute for Nuclear Researches, Dubna (USSR). Simultaneous irradiation was performed on 2 + 2 animals in a perspex cylinder, head to head. The average dose rate was 1.2 Gy/min in pulses. One cycle lasted 0.3—0.4 s and cycles were repeated every 8—10 seconds.

Experimental material: In order to compensate for the effect of random differences in relatively complex conditions during which perfusion, fixation and dissection were performed, we alternated control and experimental animals (10 rats divided into 5 groups) (Table 1). Table 1 also contains information on the radiation doses and the interval between irradiation and perfusion fixation.

The rats were anaesthetized with i.p. injections of Thiopental SPOFA. After the disappearance of pain reaction, the animals were perfused with 200—250 ml solution, the level of the latter being about 80 cm above the heart of the animals. No heparin, physiological solution, or vasodilation-inducing agents were given.

After the end of the perfusion, the animal's skull was opened and the cranial nerves and the spinal cord were dissected to remove the brain. A 1 mm thick slab was cut from the telencephalic cortex in the area corresponding to numbers 4 and 23 as parcellated by Krieg (1946), i.e. from the parietal region and the border between the anterior cingulate and the posterior cingulate. The slabs were fixed in the same solution for two hours at room temperature, and for another hour at the temperature of melting ice using osmium tetroxide.

The composition of fixing solutions: Solution I was used for perfusion, lasting about 15 min and for

Table 1. Groups of animals, radiation doses and intervals

	Intervals between the median time of irradiation and the beginning of perfusion fixation									
Hours	4	6	68.5	5	69.5	7	9	70.5	8	71.5
Rat	OS1	2S1	2L1	4S1	4L1	0S2	2S2	2L2	4S2	4L2

The first figure in the rat number indicates the dose of radiation in Gy; 0 = non-irradiated control; 2 (4) indicates a rat irradiated with 2 Gy (4 Gy). The letters indicate the interval after irradiation. S = short; L = long. Data for controls relate to the time of irradiation of rats of group 4 S. The final digit indicates the order of the animal within the group.

two-hour supplementary fixation of slabs from the brain at room temperature. It contained 1.67 mol/l formaldehyde, freshly prepared from paraformaldehyde, and 2.5×10^{-1} mol/l glutaraldehyde p.a. Reanal. Solution II contained 7.87×10^{-2} mol/l OsO_4; in both cases, the medium was phosphate buffer with an ionic strenght of 3×10^{-1} mol/l supplemented with 3×10^{-3} mol/l Ca^{2+} ions; pH = 7.4.

Preparation of slabs: After postfixation with osmium tetroxide, the tissue blocks were dehydrated with acetone and embedded in Durcupan ACM (Fluka). After polymerization semithin sections (0.3 μm) were cut from the oriented slabs (on a Pyramitome LKB) using Ultrotome III LKB, and stained with methylene blue. For ultrastructural studies, ultrathin sections were cut with a glass knife.The samples were stained with uranyl acetate and lead citrate and examined in an electron microscope Tesla BS 513.

Results

1. General characteristics of the dilation of the perivascular spaces (DPVS) and its frequency

In sections from the telencephalic cortex, generally, no interruptions of the vessel walls, of the junction of their basal membrane or the adventitia with the perivascular glial membrane, and in the adjacent regions of the astrocyte processes could be found. Some of the above structures showed infrequently a substantial edema or structural disturbances. This finding shall further be referred to as dilation of the perivascular space (DPVS).

The frequency of this phenomenon depended on three factors: the size of vessels, the radiation dose and the interval between irradiation and perfusion fixation of the tissue. It was most frequently seen after a high dose of radiation and a short interval, but although being relatively small even here. We therefore evaluated about 25 000 vessels in 19 sections from this point of view. The results are summarized in Tables 2, 3 and 4.

Table 2 contains data on the number of DPVS per 1 mm^2 of section area. This phenomenon was quite exceptionally seen in capillaries 8 μm in diameter or smaller. It was not at all observed in controls or in rats irradiated 68.5—71.5 hours previously. In capillaries with a larger diameter and thinner precapillary sections up

Table 2. Frequency of dilated perivascular spaces (DPVS) around vessels of diameter d (μm)

Group	Rat	Number of DPVS around vessels of diameter d (μm) per 1 mm²					Area of section
		$d \leq 8$	$8 < d \leq 16$	$16 < d \leq 24$	$d > 24$	Total	
0S	1	0	0.38	0.38	0	0.76	2.76
	1	0	0.34	0	1.03	1.37	2.91
	2	0	1.64	0	0.41	2.05	2.44
	2	0	0	0.35	0	0.35	2.88
	$\bar{x}$	0	0.59	0.18	0.36	1.13	2.72
2S	1	0	0.75	0.75	0.38	1.88	2.66
	1	0	3.27	0	0.65	3.92	1.53
	2	0	0.66	0.66	1.32	2.65	1.51
	2	0	2.30	2.30	0.57	5.17	1.74
	$\bar{x}$	0	1.75	0.93	0.73	3.41	1.86
2L	1	0	0	0	0	0	1.75
	1	0	0	0	0	0	1.61
	2	0	0.79	1.59	0.79	3.17	1.26
	2	0	1.74	0.35	1.04	3.13	2.88
	$\bar{x}$	0	0.63	0.49	0.46	1.58	1.85
4S	1	0.28	1.42	0.57	1.42	3.69	3.52
	1	0	1.33	0.27	1.06	2.66	3.76
	2	0.40	1.19	3.17	0.79	5.56	2.52
	2	0.45	3.64	2.73	0	6.82	2.20
	$\bar{x}$	0.28	1.89	1.69	0.82	4.68	3.00
4L	1	0.41	0	0.82	0	1.23	2.44
	1	0	0.35	0.35	0.69	1.39	2.88
	2	0	0	0.49	0.97	1.46	2.06
	$\bar{x}$	0.14	0.12	0.55	0.55	1.36	2.46

to a diameter of 16 μm, DPVS was found in all groups, with the highest incidence at a short interval following irradiation. The association with irradiation was smallest in precapillaries and postcapillary sections and other vessels with a diameter greater than 24 μm. The relatively small number of vessels with DPVS in individual categories according to section diameter indicates that our data are subject to large random differences. A comparison of overall numbers of DPVS (regardless of the diameter of the vessels) showed convincing dependence on both the radiation dose and the interval between irradiation and withdrawal; after a longer interval, the number of DPVS in irradiated rats is practically the same as in the controls.

The dependence of the occurrence of DPVS on the thickness of vessels is summarized in Table 3. In the case of capillaries with $d \leq 8$ μm, DPVS occurred

Table 3. Dependence of the occurrence of dilated perivascular spaces on the thickness of vessels

Group	Percentage occurrence of DPVS in vessels with diameter d (μm)				
	$d \leq 8$	$8 < d \leq 16$	$16 < d \leq 24$	$d > 24$	Vessels of all diameters together
0S	0	2.7	6.43	8.2	0.20
2S	0	7.9	33.2	16.6	0.62
2L	0	2.9	17.2	10.5	0.29
4S	0.054	16.5	60.4	18.6	0.85
4L	0.024	0.54	19.6	12.5	0.25

Table 4. Statistical significance of differences in the mean frequency of dilation of perivascular spaces between controls and other groups

Group	For vessel thickness d (μm)					
	$d \leq 8$	$8 < d \leq 16$	$16 < d \leq 24$	$d > 24$	$8 < d \leq 24$	Vessels of all sizes
2S	—	$P \doteq 0.2$	$P \doteq 0.2$	$P \doteq 0.3$	$P > 0.05$	$P < 0.05$
2L	—	$P > 0.9$	$P > 0.5$	$P \doteq 0.8$	$P > 0.6$	$P > 0.6$
4S	$P < 0.05$	$P \doteq 0.1$	$P > 0.05$	$P \doteq 0.3$	$P \doteq 0.05$	$P < 0.02$
4L	$P \doteq 0.3$	$P > 0.3$	$P \doteq 0.1$	$P > 0.6$	$P \doteq 0.8$	$P > 0.6$

quite exceptionally. Even after a dose of 4 Gy and a short interval only about 0.05 % of the vessels were affected. The frequency of the occurrence of DPVS increased in proportion to the diameter of vessels. Vessels with a diameter between 16 and 24 μm showed large DPVS at a short interval after irradiation with a large dose in as many as 64 % of the vessels. At longer intervals after irradiation, irradiated rats were hardly distinguishable from the controls.

Table 4 shows results of statistical analysis of the differences using t-test. The data show that, because of the low frequency of the phenomenon, the association of its occurrence with irradiation can be considered to have been demonstrated only at a short interval after irradiation with a large dose of high-energy helium ions,

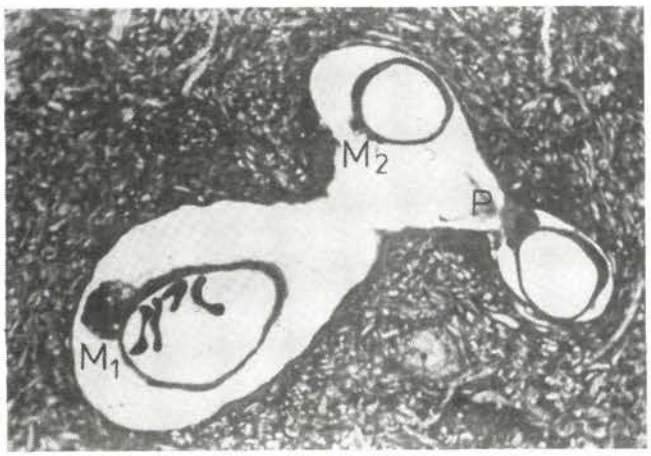


Fig. 1. Semithin section from the cerebral cortex of a rat which had been irradiated with a dose of 4 Gy helium ions 5 hours before the start of perfusion fixation. The location is from the lamina zonalis. There are three vessels in the perivascular space. A macrophage (M_1) is lying on the endothelium, another (M_2) was torn apart when the perivascular space dilated, apparently during fixation. Durcupan ACM, stained with methylene blue, magnification 1000×.

both in three categories according to the diameter of vessels and in vessels as a whole. At a shorter interval after irradiation with 2 Gy, the results are less convincing. In this case, the level of statistical significance $P<0.05$ was achieved only for vessels as a whole regardless of the diameter.

The numerical demonstration of an association between an increased frequency of DPVS in the telencephalic cortex of rats and irradiation with high-energy helium ions is insufficient for the explaination of the nature, extent or significance of this damage. We therefore shall present results of our observations of the DPVS morphology under light and electron microscopes.

2. Morphological analysis of the dilation of perivascular spaces

The extent of the dilation of perivascular spaces and its localization in the vessel wall can be estimated under light microscope. Fig. 1 shows a microphotograph (magnif. 1000×) of a semithin section from the cerebral cortex of a rat, given a dose of 4 Gy helium ions 5 hours before the start of perfusion fixation. The sample was taken from a depth of 0.75 mm below the surface, i.e. from the lamina zonalis. In the large perivascular space (with a diameter of about 70 μm in the given plane), there are three vessels, 10, 11 and 15 μm thick. The defect did not interrupt the integrity of the endothelial cells or the pericyte (P). One of the perivascular macrophages (M_1), which occurred quite frequently in our preparations, is attached

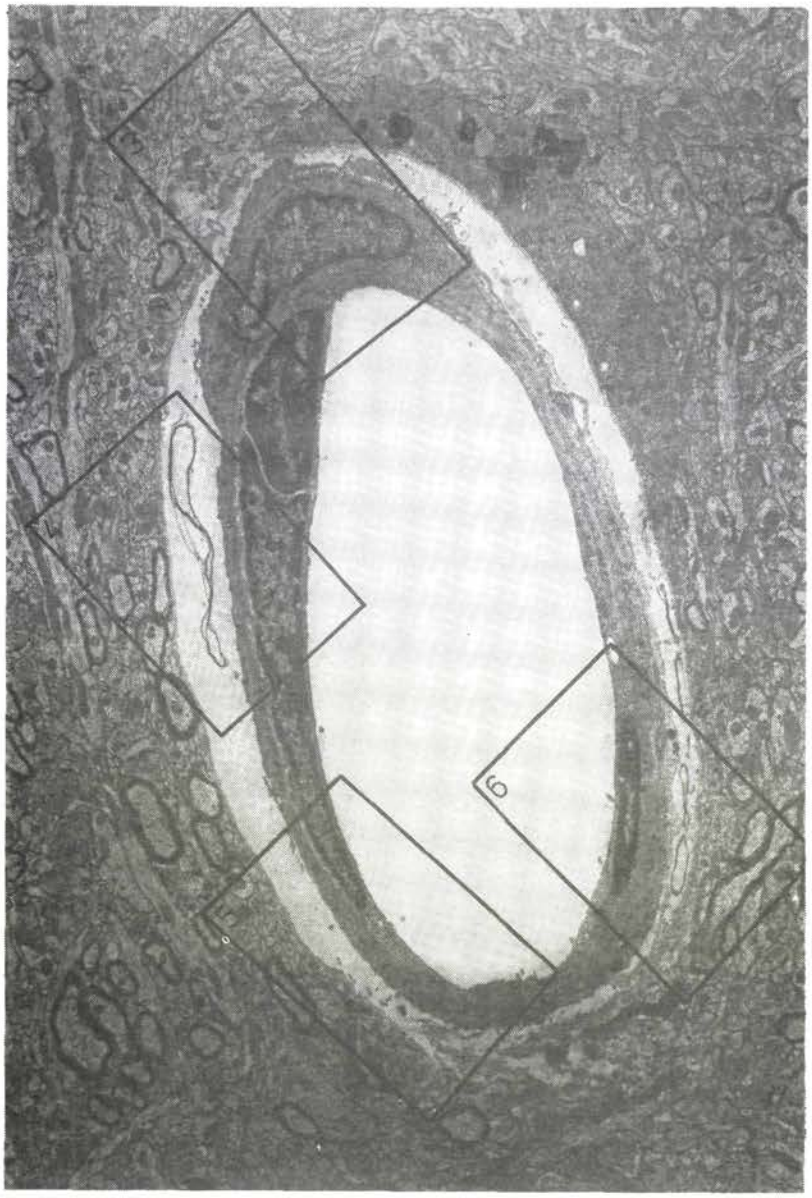


Fig. 2. Electron microgram of an ultrathin section of a capillary from the cerebral cortex of a rat six hours after irradiation with a dose of 2 Gy helium ions. The localization of the higher magnified Fig. 3—Fig. 6 is shown by frames. Magnification 7000×.

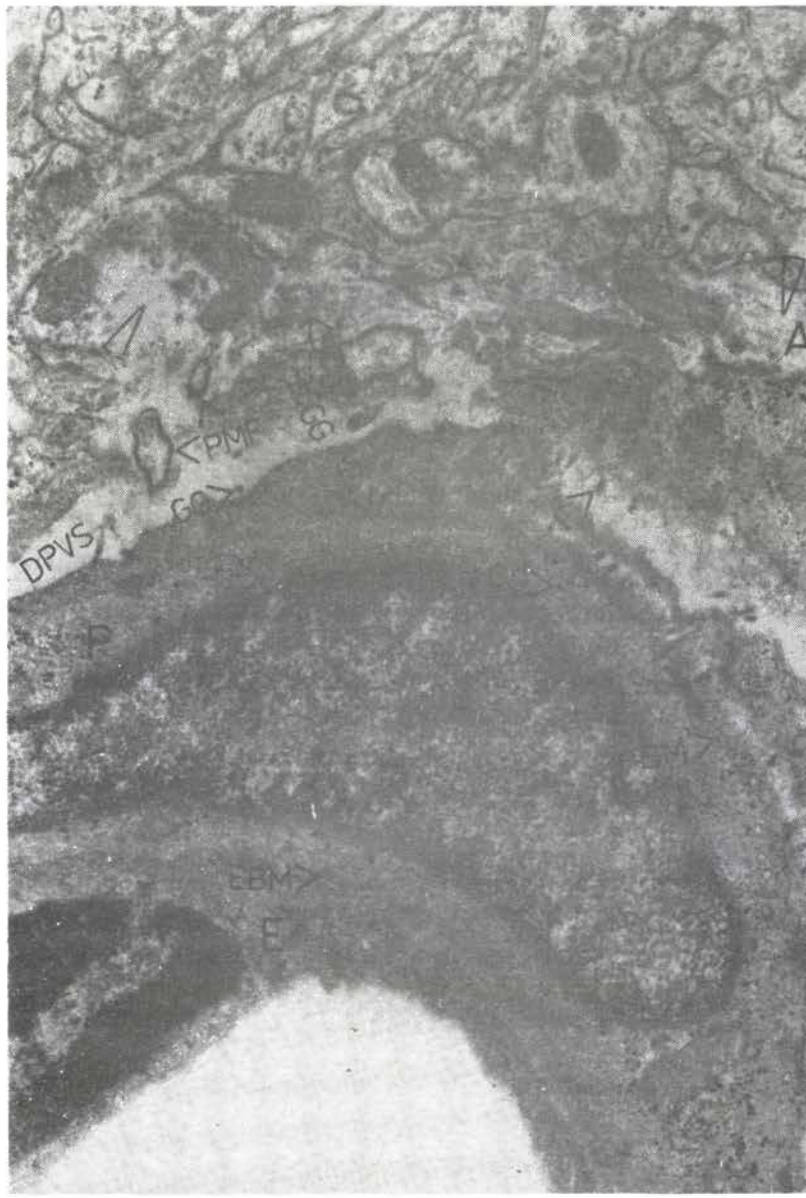


Fig. 3. Pericyte (P) and endothelium (E) showing no signs of damage, nor their basal membranes (PBM and EBM). In the dilated perivascular space (DPVS) we find grains of glycogen (GG), fibrils (F) and pseudomyeline figures (PMF). The left-hand arrow shows edematous synaptic complex (SC), the right-hand one edema of a non-myelinized axon (A). Magnification 30,000×.

to the basal membrane of the endothelium. Another macrophage (M_2) was torn apart on the dilation of the perivascular space.

According to light microscope investigation we were able to conclude that the integrity of the vascular complex and the surrounding cerebral tissue was interrupted in the vicinity of the basal membrane or the adventitia. Electron microscopy of ultrathin sections allow a much more exact localization of the defect. We shall demonstrate this using an example of a capillary from the cerebral cortex of a rat irradiated with 2 Gy helium ions 6 hours prior to fixation.

Fig. 2 (magnification 7000×) shows that the capillary in this site is formed of two endothelial cells. The dilation of the perivascular space was brought about by a defect in the astroglial process surrounding the capillary like a sleeve. The other four microphotographs (magnification 30,000×) show details of segments of the circumference of the capillary; frames in Fig. 2 show the position of the details. A large portion of the field in Fig. 3 is occupied by a pericyte. Towards the lumen of the capillary, it is covered with basal membrane with an adhering enothelial cell. All these structures are normal. The perivascular space is dilated beyond the junction between the limitans gliae perivascularis of the astrocyte process and the basal membrane of the pericyte. As suggested by numerous sections of fibrils and granules of glycogene, the area of the actual dilation contains ultrastructures typical of astrocytes. Thus the defect occurred due to a major edema of the narrow cytoplasm border of the perivascular foot of the astrocyte surrounding the capillary. The basic cytoplasm of the astrocyte and its organelles in the close vicinity of the edema do not exhibit degenerative changes or processes of necrobiosis, with only infrequent occurrence of pseudomyelin figures between fibrils released and glycogen granules. This suggests that the edema occurred shortly before the interruption of life processes in the cells by perfusion fixation, and it is probable that a certain uniformity of its features in the rest of the peripheral area of the capillary and other parts of the sections from the cerebral cortex is partly due to physicochemical changes taking place during the fixation, as shall be explained further (see Discussion).

In two places (indicated by arrows), axons are also affected by edema, one of them in a site of a synaptic complex. In Fig. 4, the neuropil shows no pathological changes, similarly as the endothelium and the cytoplasmic border of the pericyte, and the basal membrane of both cells. The width of the edema of the cytoplasmic border of the perivascular foot end reaches about 1 μm. Figures 5 and 6, from other parts of the perimeter of the capillary, show that the picture of dilation of the perivascular space, as described above, is the same. A similar situation prevailed in sections from other places of the same telencephalon preparation and in sections from the cerebral cortex of other rats showing DPVS. It was conditioned by an edema of the border of the perivascular foot of the astrocyte around the vessel, with either no or very little damage to the pericytes and to the smooth muscle cells

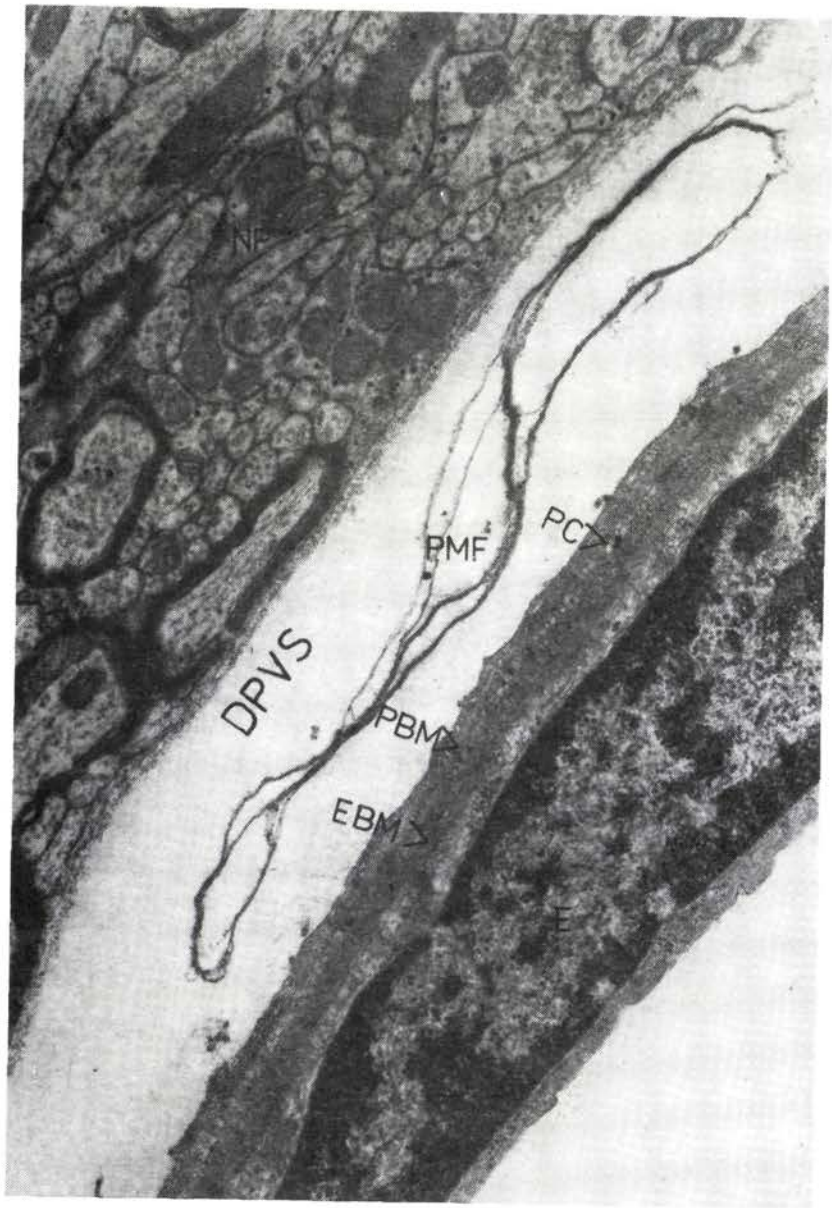
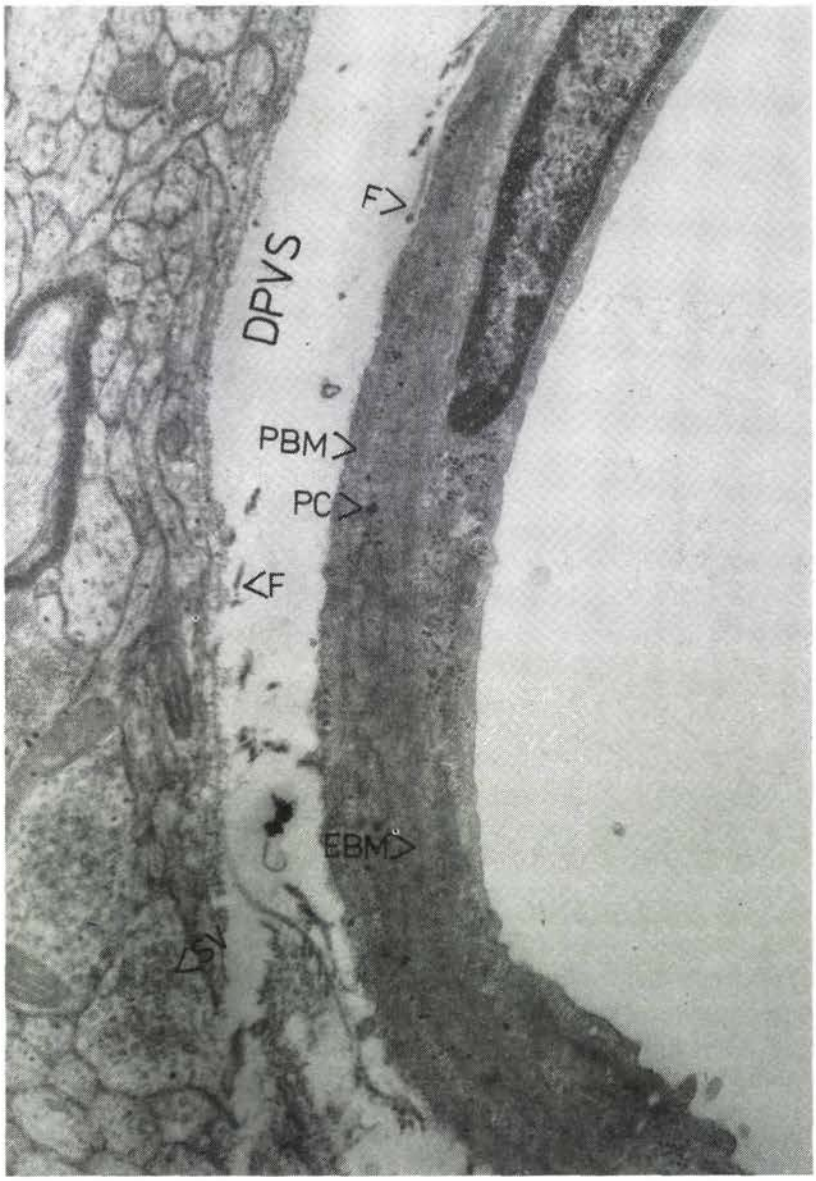
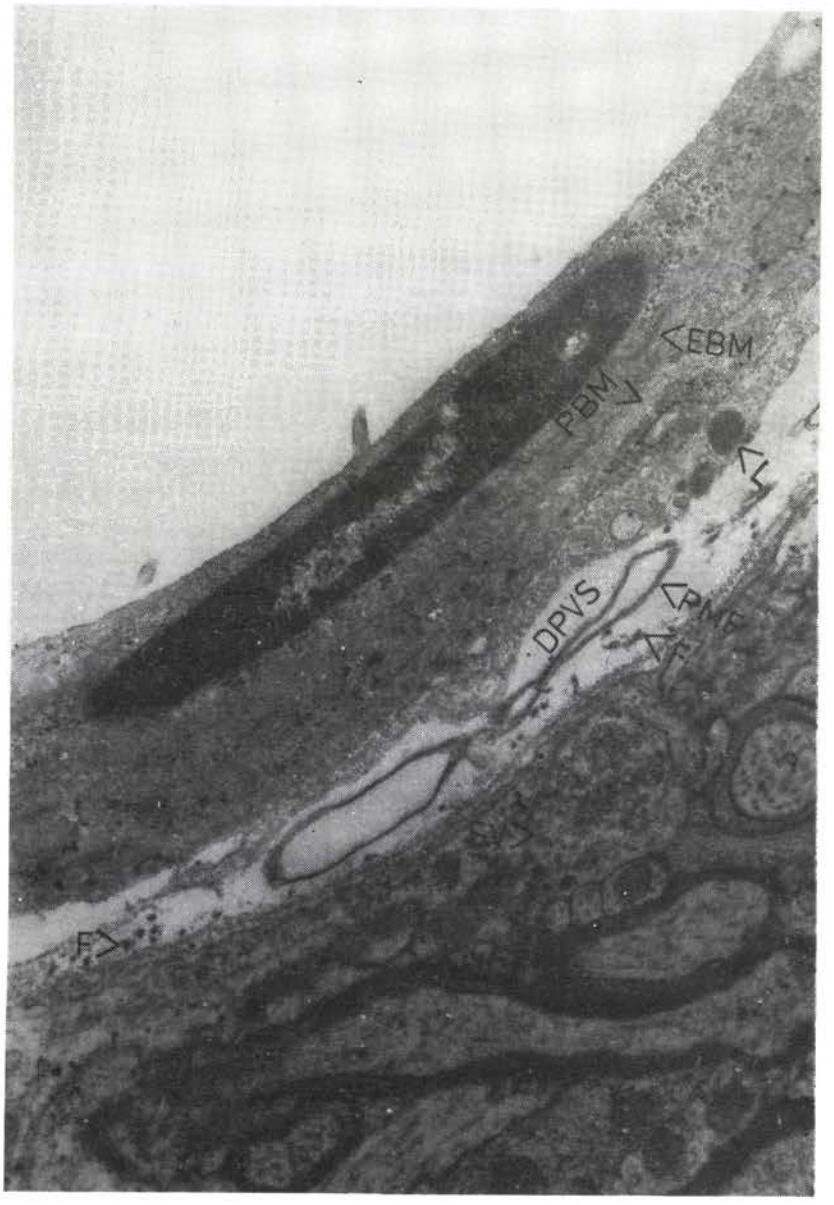


Fig. 4. A neuropil (NP), cytoplasm of the pericyte (PC), endothelium (E), basal membrane of the pericyte (PBM) and endothelium (EBM) are of normal appearance. The perivascular space (DPVS) has a width of about 1 μm. Magnification 30,000×.



Figs. 5 and 6. All ultrastructures are of normal appearance with the exception of the dilated perivascular space (DPVS), which contains the formations described in Fig. 3. Magnification 30,000×.



of larger vessels. Also, the endothelia and their basal membranes were intact except for quite rare cases.

Discussion

We shall consider our findings from three main points of view: 1. we used relatively small doses of radiation; 2. the only demonstrable difference in the morphology of vessels of the telencephalon cortex was an increased frequency of dilation of perivascular spaces in irradiated animals. The difference as compared to controls was only quantitative; 3. animals were irradiated with helium ions with an energy of 4 GeV/nucleon. The matter composition of these particles is identical with that of the alpha rays, although having by three orders of magnitude higher energies.

Ad 1. It is generally accepted that in normal rats irradiation of the brain with 1000 R (i.e. a dose of about 10 Gy) X-rays do not induce any morphological changes, and the life of animals is not shortened (Hopewell and Wright 1970). The paper by Ostenda (1979), referred to in Introduction, is an exception. Her experiments, however, were conducted using a histochemical procedure, there were no quantitative evaluation and no morphological picture of control brain sections was given. She demonstrated only changes in the gliovascular interphase after 3—125 hours following irradiation with 800 R of gamma rays (^{60}Co). The relative radiosensitivity of astrocytes has been stressed by many authors, e.g. Franke and Lierse (1966). However these authors used, guinea-pigs. According to these authors, the threshold dose for postirradiation histochemical changes of the guinea-pig brain is half that for the rat brain. Caveness (1977) described a massive break in the blood-brain barrier after 4—5 months following irradiation of the right occipital lobe of a monkey brain with a dose of 3500 rad. Irradiation of the whole brain with a dose of 1000 rad had no effect. Carmine and Richardson (1962), who emphasized the importance of vascular pathology in analysing the response of the CNS to irradiation, also found no histopathological changes in adult animals after exposures below 1000 R. Berdjis (1960) observed some sclerotic changes in the arteries of the kidneys and testes of rats one year after an exposure to 500—1000 R; the cerebral and peripheral arteries were relatively radioresistant.

Ad 2. In analysing the damage to the CNS by small doses of corpuscular radiation, insufficiently known interactions with matter and biological effects necessarily bring about difficulties in explaining the morphological findings. E.g. Haymaker et al. (1970) when looking for thindowns after cosmic rays, found narrow tracks which also occurred in the cerebral cortex of control monkeys that had not been exposed to cosmic radiation; they were similar to those found in experimental animals after high-altitude balloon flights.

For a long time morphologists have been finding both perivascular spaces in cerebral tissue and perineuronal spaces from Pestalozzi (1849), Virchow (1851) and Robin (1859) up to the 1940'ies, and they have been given various physiological significance, e.g. by Weed (1923, 1938). It was only Patek (1944) and other authors who began questioning their existence. Rosen et al. (1967) could show, by

an improved fixation technique that these were fixation artifacts. The ultrastructure of the cerebral vessels and the spaces around them were precisely described by Frederickson and Low (1969); they showed that no free perivascular space exists in the brain. The reason why the erroneous interpretation was persisting as long is that the highly hydrated astrocyte cytoplasm is difficult to preserve even with the best fixation procedures available. Edema of cytoplasm is the principle of the artificial perivascular space.

Exceptionally occurring dilations of the perivascular spaces in the cerebral tissue of control animals, which was correctly fixed by perfusion with a mixture of formaldehyde and glutaraldehyde of a suitable ionic strength and pH, postfixed with OsO_4, dehydrated and embedded in epoxy resin, can, in our opinion, be explained by the presence of disperse sites characterized as the "weak points", which give rise to local and limited dilation of the perivascular space through the synergic effect of the experimental trauma, particularly the physicochemical processes of fixation and dehydration. However, such artifacts are less likely to occur in thin vessels, such as capillaries up to a diameter of about 8 μm; we did not find any DPVS around them in our preparations from non-irradiated rats. The calculation of the occurrence of dilated perivascular spaces in all vessels regardless of the diameters showed approx. 0.2 % of vessels in our controls to exhibit the phenomenon. Apparently irradiation temporarily increased the number of suggested weak points and led to a statistically significant increase in their occurrence. This damage was reversible: at longer intervals, the frequency of DPVS was almost the same in all groups. Therefore, we consider the vascular changes which occurred under the conditions of our experiment of minor importance.

Ad 3. The final effect of the absorption of the energy of ionizing radiation is given by both the macroscopic characteristics of the process of the energy transfer to a material environment (weakening of the primary beam, absorbed dose, etc.), and the actual distribution at the microscopic level of the energy transferred (Spurný 1984). In the case of helium ions with an energy of 4 GeV/nucleon, the distribution is partially different from that with X or gamma rays. These forms of radiation transfer their energy on passing through matter by excitation, ionization, Compton process and the formation of electron-positron pairs. In dependence on the energy of X and gamma rays and the elemental composition of the matter (tissue) the processes mentioned come into play to a greater or lesser extent. In the vast majority of experiments on animals, the energy of X or gamma rays is such that the linear energy transfer (LET) is between 0.3 and 3 KeV/μm tissue and the microloci, in which there is an interaction with mater and damage to the tissue, are dense and homogenously distributed over the volume irradiated. In the case of irradiation with helium ions with an energy of 4 GeV/nucleon, the nature of the interaction with the tissues is more complex. Its LET is small in relation to the

enormous energy (speed) of these particles, only about 0.86 KeV/μm, thus lying within the range of LET of X and gamma rays as normally applied. Under the conditions of our experiment, however, there was also secondary radiation: in dependence on the localization of the area in question, this involves up to about 20 % of the dose absorbed. This secondary radiation is formed by densely ionizing particles: about 3 % protons and about 0.6 % carbon nucleons with an energy of 100 MeV/nucleon or less (Portman 1983, personal communication). In exceptional cases, there is also a nuclear reaction giving rise to heavy ions with a high LET.

In studying the consequences of irradiation with helium ions with an energy of 4 GeV/nucleon we must, therefore, suppose the existence of a wide-spectrum effect of electrons, similarly as in the case of X or gamma irradiation. Published data show that after irradiation with doses of 2 or 4 Gy of X or gamma rays there are no major morphological changes in the CNS blood vessels and their vicinity; we also found no changes in most cases after irradiation with 2 or 4 Gy of helium ions. As mentioned above, on their interaction with matter there occur microloci hit by densely ionizing secondary radiation and heavy particles released in occasional nuclear reactions. A dose of high-energy helium ions has no effect on the degree of damage within the given microloci since this is dependent on the nature of the secondary radiation and the nuclei. The radiation dose affects only the frequency of the occurrence of these microloci. This conclusion is in close agreement with our observation: we found quite exceptional sections of otherwise normal vessels with a dilated perivascular space. The frequency of the dilation was dependent on the dose and was only a temporary event. After a longer interval — about three days — repairs seemed to take place and the frequency of DPVS was the same in irradiated and control animals.

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Abbreviations

EBM	endothelial basal membrane
PBM	pericyte basal membrane
PC	pericyte cytoplasm
DPVS	dilated perivascular space
E	endothelia
F	fibril
GG	glycogen granulum
L	lysosome
M	macrophage
ML	membrana limitans

NP neuropil
P pericyte
PMF pseudomyeline figure
SC synaptic complex
SV synaptic vesicule

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