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# Stases are associated with blood–brain barrier damage and a restricted activation of coagulation in SHRSP

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ABSTRACT

Cerebral small vessel disease (CSVD) is a chronically proceeding pathology of small brain vessels associated with white matter lesions, lacunar infarcts, brain atrophy and microbleeds. CSVD leads to slowly increasing cognitive and functional deficits but may also cause stroke-like symptoms, if vessels in critical brain areas are affected. Spontaneously hypertensive stroke-prone rats (SHRSP) exhibit several vascular risk factors, develop infarcts and hemorrhages and therefore represent a relevant model for the study of CSVD. Using this animal model, we recently demonstrated that intravasal accumulations of erythrocytes, we interpreted as stases, stand at the beginning of a pathological vascular cascade. After stases microbleeds occur, which are followed by reactive microthromboses. Bleeds and thromboses finally cause hemorrhagic infarcts. Immunohistochemical stainings show, that plasma proteins like IgG are deposited in the walls of vessels affected by stases. Further, we found small clots and thread-shaped aggregations of thrombocytes as well as thread-shaped structures of von Willebrand-Factor within stases. Thus, we conclude that blood–brain barrier damages occur in the neighborhood of stases and stases seem to be associated with a restricted activation of blood coagulation without formation of obstructive thromboses. Finally, we demonstrate that small vessel damage rarely appears in the cerebellum. Even animals with multiple cerebral infarcts may be free of any cerebellar vascular pathology.



## 1. Introduction

Human cerebral small vessel disease (CSVD) is not only associated with acute cerebral ischemia [1] but has also been recognized as one major etiology of vascular dementia [2]. Human CSVD is associated with some well established imaging features (including white matter lesions, lacunar infarcts, brain atrophy and microbleeds/microhemorrhages [3]), whereas its underlying histology has been sparsely described systematically [4–6]. The main histopathological findings related to the white matter and basal ganglia's small arteries include "disorganized arterial lesions" (in part with deposits of fibrinoid material and local enlargement of the vessel), concentric hyaline vessel wall thickening with concomitant luminal narrowing (in part caused by plasma protein leakage into the vessel wall), enlarged perivascular spaces, aneurysm formation implying a dilatation of the vessel lumen and extravasations of blood (partially through a damaged vessel wall) [4–6].

In a former study we aimed to identify, whether the vascular histopathology associated with CSVD proceeds in definite stages. By means of serial histological investigations of spontaneously hypertensive stroke-prone rats (SHRSP) – an animal model accepted as potentially relevant to the study of CSVD [7,8] – we were able to describe a chronology of definite pathological stages of the cerebral small vessel disease [9]. Thereby our results demonstrate that the vascular pathology in SHRSP is initiated by an accumulation of erythrocytes in capillary and arteriolar segments of different brain regions [9]. We parsimoniously interpreted those accumulated erythrocytes as stases. Meanwhile we have examined the etiology of the erythrocyte accumulations more closely; thereby our further data support the relevance of stases for the initial phase of CSVD. Moreover we were interested in the correlation between the vascular histopathology in the cerebrum and the cerebellum. Our results reveal that the cerebellum is rarely affected by small vessel changes.

## 2. Material and methods

Animal procedures were conducted after obtaining the approval of the Animal Care Committee of Sachsen-Anhalt (reference number of license for animal testing 42502-2-943, July 2009, Magdeburg, Sachsen-Anhalt). Animals were housed with a natural light–dark cycle and allowed to access water and food ad libitum. Briefly, ninety four male SHRSP (Charles River Laboratories International, Inc.,

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Wilmington, MA, USA) were investigated histologically (12 weeks [w] n = 6, 14 w n = 5, 16 w n = 3, 18 w n = 4, 20 w n = 2, 22 w n = 2, 24 w n = 5, 26 w n = 3, 28 w n = 11, 30 to 32 w n = 15, 34 w n = 2, 36 w n = 15, 40 w n = 12, 41 to 42 w n = 3, 44 w n = 6). Thirty seven Wistar rats at corresponding ages served as control group (12 w n = 4, 18 w n = 4, 26 w n = 4, 32 w n = 5, 36 w n = 7, 40 w n = 6, 65 w n = 2, 2 years [a] n = 3, 3 a n = 2). Rats were transcardially perfused with 120 ml phosphate-buffered saline (PBS) followed by 120 ml 4% paraformaldehyde (PFA) within 4 min. Brains were removed, stored in 4% PFA for 48 h, placed for cryoprotection into 30% sacharose for 6 days, and frozen in methylbutane at −80 °C. Coronal slices (30 μm) of the whole brain were prepared with a cryotoma (Leica, Nussloch, Germany) and stained with hematoxylin/eosin (HE) (for details see [9]). All brains of SHRSP and controls were investigated for the occurrence of vessels with accumulated erythrocytes, microbleeds and microthromboses in the basal ganglia, cortical regions, corpus callosum, and hippocampus [9]. For the cerebellum a sample of brains (32 SHRSP, 11 controls) was examined. Brain slices were immunohistochemically stained with STL (Solanum Tuberosum Lectin, Vector Laboratories, Burlingame, USA, 1:500; binds on glycoproteins on the surface of endothelium cells [10]), anti-von Willebrand-Factor (vWF) (abcam, 1:200) and a thrombocyte antibody (Fitzgerald Industries International, 1:1000). Slices were washed anew before application of anti-rat-IgG-Cy3 or anti-rabbit-IgG-Cy3 for 2 h at room temperature and subsequent DAPI-staining. As a control first antibodies were omitted during immunohistochemistry staining. Cy3-donkey-anti-rat-IgG was used for detection of IgG and Cy3-donkey-anti-rabbit-Cy3 was used as secondary antibody for anti-vWF- and anti-thrombocyte-antibody. IgG was examined in 9 SHRSP (18 w n = 1, 24 w n = 1, 28 w n = 2, 32 w n = 2, 34 w n = 1, 36 w n = 2) vWF in 12 SHRSP (20 w n = 1, 22 w n = 1, 24 w n = 1, 28 w n = 3, 32 w n = 1, 34 w n = 1, 36 w n = 2, 40 w n = 1, 42 w n = 1) and 2 controls ( 26 w n = 1, 2 a n = 1), thrombocytes in 2 SHRSP (28 w n = 1, 34 w n = 1) and 1 control (26 w n = 1).

### 2.1. Chronological progress of the vascular histopathology in SHRSP

As recently reported, we see an age-dependent cascade of vascular changes in SHRSP [9]. The first detectable changes are intravasal accumulations of erythrocytes, which we refer to as stases. Starting from an age of 28 weeks virtually all investigated SHRSP show such stases. The stases primarily occur in capillaries (Fig. 1A), but can be found later on also in arterioles (Fig. 1B and C). A comparison of 28 week old SHRSP with animals aged 12 weeks reveals that single stases become bigger in their extension occur more frequently in arterioles (Fig. 1B and C) and the number of stases per animal increases dramatically [9]. Arterioles with stases frequently show enlarged perivascular spaces (Fig. 1C). Microbleeds (Fig. 1D) which may occur according to our last data already at an age of 24 weeks most likely initiate the actual infarct state. Microthromboses obviously develop as a reaction to these microbleeds. Therefore small vessel occlusions are frequently seen to be surrounded by fresh and older hemorrhages (Fig. 1E and F). Both, hemorrhages and small vessel occlusions are located in infarct regions of SHRSP aged 32 weeks or older. The resultant necrosis and spongiform tissue damage represent the terminal stage of the pathological cascade (Fig. 1E and F).

In the control group we also found intravasal erythrocyte accumulations, which could be hardly detected due to their overall low number per brain, their restricted extent and their predominant occurrence in capillaries. Moreover, significantly fewer animals (54%, 20 of 37) were affected compared to the SHRSP group (91%, 86 of 94). None of the controls exhibited hemorrhages or infarctions.

### 2.2. Blood–brain barrier (BBB) damage and thrombocyte aggregation in the vicinity of stases

Stases are visibly after immunohistochemical staining by the frequently occurring autofluorescence of the erythrocytes (Fig. 2).

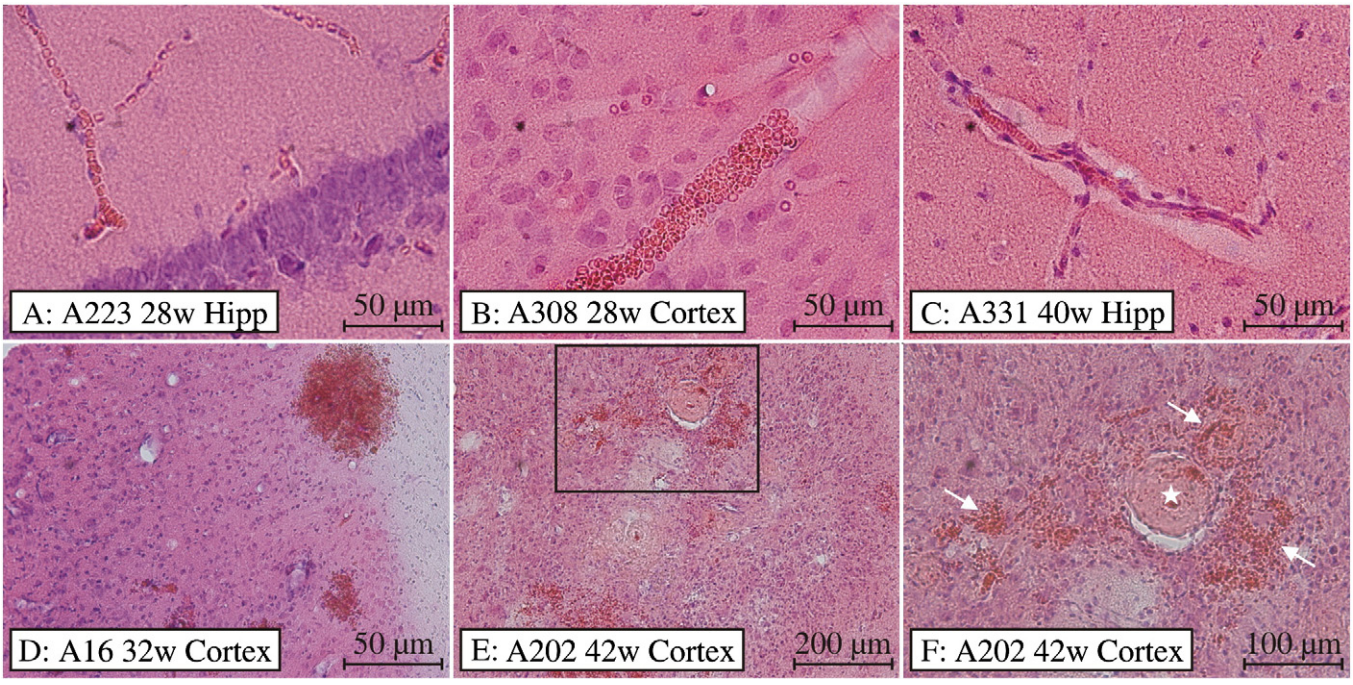


**Fig. 1.** Pathological cascade of the cerebral small vessel disease in SHRSP. Accumulations of erythrocytes in capillaries (A) and arterioles (B), we referred to as stases, represent the first step (A–C). Note the enlarged perivascular space in C. Later on microbleeds occur (D) which obviously initiate the phase of infarcts illustrated in E and F. Most likely, small vessel occlusions (asterisk; F) develop as a consequence of bleedings (arrows; F). Both, microbleeds and microthromboses cause the ischemic damage visible as spongy like tissue destructions (E). Panel F is a higher magnification of E.