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Studies of free radical generation by neurons in a rat model of cerebral venous sinus thrombosis

Amit Kumar Srivastava [a], Jayantee Kalita [a], Preeti Dohare [b], Madhur Ray [b], Usha Kant Misra [a,*]

[a] Department of Neurology, Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Raebareily Road, Lucknow 226014, India
[b] Pharmacology Division, Central Drug Research Institute, Lucknow, India

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ABSTRACT

The role of free radicals in the pathogenesis of arterial stroke is well documented but not in venous stroke. The aim of the study was to investigate the possible role of free radicals in the pathogenesis of cerebral venous sinus thrombosis (CVST). For inducing CVST in Sprague–Dawley rats, a cranial window was made to expose the superior sagittal sinus (SSS). On the exposed sinus, a strip of filter paper soaked with 40% ferric chloride was applied. In the control rats 0.9% saline was used instead of ferric chloride. After induction of sinus thrombosis, clinical evaluations were done on days 1, 2 and 7 for neurological deficit, weight of thrombus and brain lesion volume. In neuronal-rich cell preparations flow cytometric estimations were done at different time points. In the study group on sequential follow-up, there was spontaneous recanalization of SSS as well as a significant decrease in brain lesion volume. An insignificant improvement in neurological deficit was also observed. In the controls, there was no neurological deficit or evidence of infarction. Neuronal free radical levels were significantly increased in the study group on day 1 compared to controls, but on follow-up free radicals levels decreased. It is concluded that the free radicals increase in the early stage of venous stroke and may be important in its pathogenesis.

Cerebral venous sinus thrombosis (CVST) is an important cause of stroke in young people, and common in India, especially after pregnancy. In an earlier study we reported that the spectrum of venous thrombosis was same as in the West because of a paucity of perpurial (postpartum) CVST patients in our study [12]. The venous sinus thrombosis results in infarction, hemorrhagic transformation and rarely intracranial hemorrhage because of the occlusion of major sinuses or cortical veins. Proximal to the occlusion, there is venous congestion resulting in increased pressure, diapedesis of red blood cells and hemorrhagic changes. The prognosis of venous sinus thrombosis is better than arterial because of the difference in the temporal profile of venous infarction which is slower and richer in venous collaterals than in arteries.

Hypoxia results in a sequence of events including generation of cytokines, chemokines, free radicals and excitotoxicity [2]. These abnormalities have been well documented in the arterial ischemic stroke [1,2,6,10,14,17], however there is a paucity of such studies in venous sinus thrombosis. We have modified the animal model of CVST reported by Rottger et al. [19], by creating a small window over the superior sagittal sinus (SSS) and applying 40% ferric chloride which is less invasive and closer to human CVST [23]. We have used this model to study spontaneous recanalization and generation of free radicals and to understand the pathogenesis of the CVST.

Forty-eight male Sprague–Dawley rats weighing 210–285 g obtained from Animal House, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow were used in the study. The rats were divided into two groups: the study group comprised 36 rats in which thrombus were induced; and a sham-operated control group comprised 12 rats. The study group was further divided into three subgroups of 12 rats each and analyzed on days 1, 2 and 7 after the induction of thrombus. 2,3,5-Triphenyltetrazolium chloride (TTC) staining was performed to study the volume of infarction/ischemia and the thrombus weight. These were measured at each time point in six rats from each group. The neuronal level of reactive oxygen species (ROS), nitric oxide (NO), peroxinitrite ($OONO^-$), Ca^{2+}, mitochondrial membrane potential ($\psi\Delta m$) and necrotic/apoptotic cell population were analyzed in the rest of the rats. In the control

Abbreviations: CVST, cerebral venous sinus thrombosis; SSS, superior sagittal sinus; TTC, 2,3,5-triphenyltetrazolium chloride; ROS, reactive oxygen species; NO, nitric oxide; $OONO^-$, peroxinitrite; Ca^{2+}, calcium ion; $\psi\Delta m$, mitochondrial membrane potential; DCDHF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DCF, 2′,7′-dichlorofluorescein; DAF-2DA, diaminofluorescein diacetate; DAF-2T, diaminofluorescein-2T; DHR 123, dihydrorhodamine 123; Rh 123, rhodamine 123; FITC, fluorescein isothiocyanate; ATP, adenosine triphosphate; MRI, magnetic resonance imaging.

* Corresponding author. Fax: +91 522 2668017.
E-mail addresses: drukmisra@rediffmail.com, ukmisra@sgpgi.ac.in (U.K. Misra).

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group, the above-mentioned parameters were also analyzed. Rats were maintained on alternating 12 h light and dark cycle with free access to water and food. The study was approved by the local ethics committee, and all experiments were carried out in accordance with the institutional guidelines on the care and use of experimental animals.

For inducing the thrombosis of the SSS, the rat was anesthetized with chloral hydrate (350 mg/100 g body weight i.p.) and fixed in a stereotactic frame. Its skull was exposed by a 1.5 cm midline skin incision at the dorsal aspect of the head. A cranial window (10 mm × 1.5 mm) was made to expose the SSS on which a small strip of filter paper soaked in 40% ferric chloride was applied in the dark. After 5 min, the strip of ferric chloride was removed and the field was flushed with 0.9% saline. The wound was closed and the skin was sutured. During the surgical procedure, the body temperature was maintained at 37 °C with the help of a thermostatically controlled heating pad. The rat was kept in the cage and allowed free access to food and water. Sham-operated controls underwent a similar operative procedure except that 0.9% saline was used instead of ferric chloride [23].

All the rats were subjected to a neurological evaluation 1 day prior to and after the 1st, 2nd and 7th day of the surgical procedure using a score (0 = no observable neurological deficit (normal); 1 = failure to extend left forepaw on lifting the whole body by tail (mild); 2 = circling to the contralateral side (moderate); 3 = leaning to the contralateral side at rest or no spontaneous motor activity (severe) [9].

The rats were decapitated, the brain was removed and placed in ice-cold saline for 5 min and then cut into seven 2 mm coronal slices. The brain slices were incubated in 2% TTC dissolved in saline for 15 min at 37 °C. The stained brain sections were stored in 10% formalin and refrigerated at 4 °C. The decolorized area in each brain slice was determined using public domain software (Biovis, Expert vision, India). The brain lesion volume was calculated by the product of average slice thickness (2 mm) and the sum of infarction area in all the seven slices. The results were expressed as mean ± S.D.

The thrombus formed was weighed immediately after isolation; its weight was denoted as wet weight. The same thrombus was placed on a plastic surface for 15 min, 15 cm below a 60 W heating lamp, following which its partially dry weight was determined. Then the partially dry thrombus was dried in an oven for 1 h at 60 °C to a constant dry weight. The thrombus weight reported in this study is constant dry weight. Data were expressed as mean ± S.D.

The forebrain neurons were isolated according to the method of Oyama et al. [16] further modified by Dohare et al. [3].

Finely chopped forebrain tissue was treated with dispase (1000 protease unit/ml) for 45 min at 37 °C. After enzymatic treatment, the dissociated neurons were passed through a filter (mesh diameter ~53 μm) to remove large neurons and tissue fragments. HEPES–tyrode solution (145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 5 mM HEPES, pH 7.4) was used to prepare homogenous suspension of individual neurons. A viability test of neuronal cell population was done using propidium iodide (PI) resulting 90% of the viable homogeneous population. Neurons were gated on the basis of forward and side-scattered during acquisition for further study. All the parameters were analyzed within the neuron gate.

The ROS level was measured with the help of the 2′,7′-dichlorodihydrofluorescein diacetate (DCDHF-DA), which is cell-permeable and non-fluorescent. In the presence of ROS it oxidizes inside the cells producing a fluorescent compound, 2′,7′-dichlorofluorescein (DCF), which remains trapped within the cell.

Isolated neurons (10^6/ml) were incubated with 50 μM 2,7-DCDHF-DA for 30 min at 37 °C, and washed twice in HEPES–tyrode buffer. The fluorescence was monitored in a flowcytometer (FACS Calibur, Becton Dickinson, UK) at an excitation wavelength of 488 nm and emission 530 nm [18]. For each sample, 10,000 cells were acquired. Data were analyzed using Cell Quest Software (Becton Dickinson, UK) and expressed as an average of fluorescence intensity of analyzed cell population (mean fluorescence intensity ± S.E.M.).

Diaminofluorescein diacetate (DAF-2DA) is a non-fluorescent cell-permeable dye. Inside the cell diacetate groups on the DAF-2DA reagent are hydrolyzed by cytosolic esterases releasing DAF-2 and sequestering the reagent inside the cell. NO converts this non-fluorescent dye, DAF-2, to its fluorescent triazole derivative, DAF-2T.

For NO level estimation, neurons (10^6/ml) were incubated with 10 μM 4–5, DAF-2DA for 30 min at 37 °C and fluorescence was determined in a flowcytometer (FACS Calibur, Becton Dickinson, UK) at excitation wavelength 488 nm and measuring emission at 515 nm [3]. For each sample, 10,000 cells were acquired. Data were analyzed using Cell Quest Software (Becton Dickinson, UK) and expressed as an average of fluorescence intensity of analyzed cell population (mean fluorescence intensity ± S.E.M.).

The level of $ONOO^-$ was measured by the $ONOO^-$ dependent oxidation of dihydrorhodamine 123 (DHR 123) to fluorescent rhodamine 123; this measurement was based on the principles of the method by Kooy et al. [13].

Isolated neurons (10^6/ml) were incubated in 5 μM DHR 123. After incubation for 60 min at 37 °C, the fluorescence was measured in a flowcytometer (FACS Calibur, Becton Dickinson, UK) at excitation wavelength of 500 nm and at emission wavelength of 536 nm [3]. For each sample, 10,000 cells were acquired. Data were analyzed using Cell Quest Software (Becton Dickinson, UK) and expressed as an average of fluorescence intensity of analyzed cell population (mean fluorescence intensity ± S.E.M.).

Rhodamine 123 (Rh 123) is a lipophilic fluorescent dye, which selectively locates mitochondria in the living cells. The alterations in the mitochondrial membrane potential were measured by the retention of rhodamine 123, which accumulates electrophoretically into energized mitochondria in response to their negative inside membrane potential.

Isolated neurons (10^6/ml) were incubated with Rh 123 (1 μg/ml) for 30 min at 37 °C. The neurons were washed twice and resuspended in HEPES–tyrode buffer and changes in the membrane potential were observed in a flowcytometer (FACS Calibur, Becton Dickinson, UK) at an excitation wavelength of 488 nm and emission wavelength of 530 nm [5]. For each sample, 10,000 cells were acquired. Data were analyzed using Cell Quest Software (Becton Dickinson, UK) and expressed as an average of fluorescence intensity of analyzed cells population (mean fluorescence intensity ± S.E.M.).

Fluo-3AM is a membrane-permeable dye. On being taken up by the cells, the ester is hydrolyzed by an esterase in the cytoplasm to release the free acid (hydrolyzed) form of the dye (Fluo-3), which is sensitive to calcium. The fluorescence of Fluo-3 increases on calcium binding.

Isolated neurons (10^6 cells/ml) were washed twice with PBS (pH 7.4) without calcium and magnesium. After washing, the cells were suspended at 2×10^6 cells/ml in PBS and incubated for 30 min at 37 °C with 100 μg/ml Pluronic F-127 and 7.5 μM of Fluo-3AM. Fluorescence was observed in the flowcytometer (FACS Calibur, Becton Dickinson, UK) at an excitation wavelength of 506 nm and emission wavelength of 526 nm [16]. For each sample, 10,000 cells were acquired. Data were analyzed using Cell Quest Software (Becton Dickinson, UK) and expressed as an average of fluorescence intensity of the analyzed cells population (mean fluorescence intensity ± S.E.M.).

The isolated neurons (10^6 cells/ml) in the binding buffer were treated with FITC-labeled annexin-V and PI according