Journal of Research in Clinical Medicine

J Res Clin Med, 2020, 8: 25
doi: 10.34172/jrcm.2020.025
https://jrcm.tbzmed.ac.ir

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Original Article

Destructive effect of digitalis overdose on blood-brain barrier in rats; an experimental study

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Article info

Article History:
Received: 1 May 2020
Accepted: 7 June 2020
e-Published: 12 July 2020

Keywords:
- Digitalis toxicity
- Blood-brain barrier
- Rat brain
- Digoxin

Abstract

Introduction: Cardiac glycosides are widely used in critical cardiac diseases despite their unexplained mechanisms on cardio-respiratory system and other autonomic complications within both intra-uterine and post-natal life. The aim of this study is to investigate if digitalis overdose could cause a result in such complications by a destructive effect on blood-brain barrier (BBB).

Methods: Twenty-five male Sprague Dawley rats weighing 300–350 g were divided into the following groups: control (n=5), sham (isotonic) (n=5), therapeutic dose (n=5), arrhythmogenic dose (n=5), and lethal dose (n=5). The animals were euthanized and their brains were extracted. The brains were histopathologically and immunohistochemically examined to evaluate BBB morphology in the superior temporal cortex.

Results: One animal died because of experimental procedures on the first day. Macroscopic examination of brains revealed brain edema, subarachnoid hemorrhage (SAH), and narrowed cisterns in toxic doses of digitalis-treated animals. Brain histopathological examination of these groups revealed bloody subarachnoid and cisternal spaces, cortical arteriolar vasospasm, neurodegeneration, and even peri-arteriolar neuroglial component fragmentation; these changes induced BBB destruction in the high-dose digitalis-treated animals.

Conclusion: Digitalis should not be used with overdoses if the cardio-respiratory arrhythmia is unexpectedly appearing in low doses against the possibility of defected or disrupted BBB.

Introduction

Digitalis should be used with caution because of its irreversible toxicological effects on multiple organs,[1] especially newly described subarachnoid hemorrhage (SAH) and blood-brain barrier (BBB) destruction. Toxic doses of digitalis cause hemorrhagic necrosis of the intestine.[2] They also have neuro-necroptotic and congenital effects because they easily pass through the BBB and placenta.[1] Excessive vagal stimulation-induced fatal bradycardia is a dangerous complication[3] because of central pontine myelinolysis.[4] Accidental poisoning frequently occurs in children.[5] Digoxin antibodies have a vasoconstrictor/hemorrhagic effect on cerebral arteries. Digitalis toxicity could result in intestinal dysfunctions affecting the neurenteric network.[6] Encephalopathy[7] and fulminant hepatic failure[8] have also been reported with toxic doses.

The most affected parts of the nervous system are chemoreceptors and baroreceptor networks[9]; central, autonomic, and peripheral nervous system; choroid plexus; neurohypophysis; adenohypophysis; area postrema; superior cervical sympathetic ganglion; and adrenal medulla.[10] Hippocampal injury is possible after digoxin treatment.[11] Glycosides are transported to the cerebrospinal fluid via choroidal arteries in patients with SAH.[12] BBB destruction increases BBB permeability[13]and vasospasm-induced cerebral ischemia results in passive dilation of cerebral vessels during BBB destruction.[14] Therefore, digitalis may be more dangerous in such patients. The hyperthermic effect of digoxin causes acute thermal BBB destruction.[15] BBB destruction facilitates demyelinating[16] and neurodegenerative disease.[17] It

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can decrease neuroimmunity,[17] increase autonomic imbalances,[9] and cause acute lethal anaphylaxis.[18] The placental transition of digoxin can be very dangerous for fetal brain development[19] probably disrupted BBB.

Materials and Methods

Animals

Twenty-five male Sprague Dawley rats weighing 300–350 g were used. The rats were kept in a temperature-controlled (22–24°C) room with a 12-hour light/12 hour dark cycle during the study. They were fed a standard laboratory diet via ad libitum. The rats were randomized into five groups, each consisting of five rats: control, sham, treatment dose, arrhythmogenic dose, and lethal dose.

Study design

Before starting our experimental study, a preliminary study was conducted to detect the dosage of the digitalis for all groups. The doses in the literature for the treatment, arrhythmogenic dose, and lethal doses were different in many studies and there was no consensus.[20,21] To detect the doses for treatment, arrhythmogenic, and lethal doses, the rats were injected different doses while they were connected to the electrocardiograph (ECG), and their O_2 saturation and beats per minute were monitored. While observing the vital changes and monitoring the ECG, the arrhythmogenic and lethal effects of the doses were recorded. After the preliminary study, rats have injected digitalis doses, following the group-specific dosages.

The study injection protocol is shown in Table 1. The control group was untouched during the test period. An isotonic saline solution was given intraperitoneally to the sham group. The other three groups were administered daily digoxin injections intraperitoneally in different doses. During the first day, all digoxin injected groups were received the same dose of 1-cc digoxin. The first treatment group received 1 cc digoxin until the end of the experiment. The second (arrhythmogenic) group was received 2 cc digoxin by the second day of the study and increased to 3 cc in the second week and continued until the arrhythmia observed. When arrhythmia occurred, the rats were sacrificed. The third (lethal) group received gradually increased doses to 4-cc digoxin (heavy arrhythmic dose) by the third week. This group received 5-cc digoxin (lethal dose) on the 22nd day, and the experiment was terminated.

Biochemical and histopathological analysis

After the injection period, all rats were anesthetized with ketamine/xylazine and sevoflurane. Cardiac blood sample was taken for biochemical investigations from all the euthanized animals and the organs were placed in 10% formaldehyde for histopathological examination. Blood digoxin levels of all rats were studied by the electrochemiluminescence immunoassay method (Cobas® E601).

Table 1. Intraperitoneally Administered Digoxin Dosages Schedule

Groups	Day 0	Day 1-7	Day 8-14	Day 15-21	Day 22
Control	-	-	-	-	-
Sham (saline)	1 cc*	1 cc	1 cc	1 cc	1 cc
Therapeutic dosage (digoxin 0.33 mg/mL)	1 cc	1 cc	1 cc	1 cc	1 cc
Arytmogenic dosage (digoxin 1 mg/mL)	1 cc	2 cc	3 cc	**	-
Lethal dosage (digoxin 1.65 mg/mL)	1 cc	2 cc	3 cc	4 cc	5 cc

*1 cc = 0.33 mg/mL
** Rats were sacrificed when arrhythmia occurred

Each brain tissue section was stained with hematoxylin-eosin (H&E) and glial fibrillary acidic protein (GFAP) for the examination of the neurons and astrocytes with a light microscope. H&E staining was performed according to routine protocols.[22] Briefly, after preservation, dehydration, clearing, and paraffin infiltration procedures, 5 µm longitudinal sections were stained with hematoxylin solution for 5 minutes and then rinsed with distilled water, stained with eosin solution for 3 minutes, followed by gradual dehydration with alcohol and cleaned in xylene. GFAP staining procedure was an immunohistochemical detection of the astrocytes performed by pretreatment of 20 µg/mL proteinase K for 15 minutes. When histological slices were prepared and examined, astrocytes and periarteriolar neuronal numbers in BBB were estimated by using stereological analyses.

Statistical analysis

The total glial cells and degenerated neurons determined by histopathological examinations of the slices and the comparisons of the groups were analyzed by statistical SPSS program 25.0, one-way non-parametric ANOVA (Kruskal-Wallis test) analysis. Statistical significance was accepted as $P<0.05$, $P<0.005$, $P<0.0005$, $P<0.0001$, $P<0.00001$.

Results

Biochemical results

Digoxin doses in the blood of the groups were determined by the doses injected during the experiment. Blood biochemical results are mentioned in Table 2.

Histopathological results

No apparent macroscopic lesions were observed in the

Table 2. Blood digoxin levels of the rats after they sacrificed

Groups/Dosage	Blood Digoxin Levels
Control / 0 mg/mL	0 ng/mL
Sham / 0 mg/mL	0 ng/mL
Therapeutic / 0.33 mg/mL	1.81 ng/mL
Arrhythmogenic / 1 mg/mL	3070.5 ng/mL
Lethal / 1.65 mg/mL	4999 ng/mL

brain of control animals. The basement membranes of capillary endothelium were deformed, had fibrillary extensions and showed fewer astrocytes (Figure 1). Stereological methods of astrocyte number estimation produced with 3-D cubic and cylindric samples are mentioned in Figure 2. In high-dose digoxin groups, BBB destruction was evident in the form of ruptured basement membranes and disarranged junctional complexes between endothelial cells of arterioles and more degenerated astrocytes (Figure 3). Histopathological appearance of a normal BBB with astrocytes in a normal rat, partially destructed BBB, fragmented astrocytic feet in therapeutic dose group. The most destructed BBB fragmented astrocytic pedicles in the lethal dose given rats (Figure 4). In some animals, occluded microarterioles with desquamated endothelial and blood cells were noted. Total glial cell ratio/degenerated neuron ratio in BBB was as follows: Control 2950 ± 513/15 ± 4, Sham 2910 ± 390/16 ± 4, therapeutic dosage 2890 ± 215/20 ± 6, arrhythmogenic dosage 2360 ± 480/218 ± 51, lethal dosage 1760 ± 250/570 ± 98. Total glial cell ratio/degenerated neuron ratio in BBB, and statistical results are shown in Tables 3 and 4, respectively.

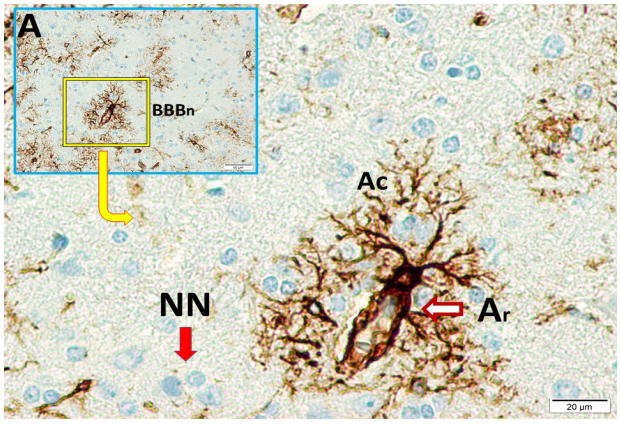

Figure 1. Histological appearance of normal BBB (BBBn) (LM, GFAP, x20/A). The magnified appearance of BBBn in cerebral arteries (Ar) and astrocytes (Ac) around the arterioles (Ar) (NN) (LM, GFAP, ×40/Base) in a normal rat.

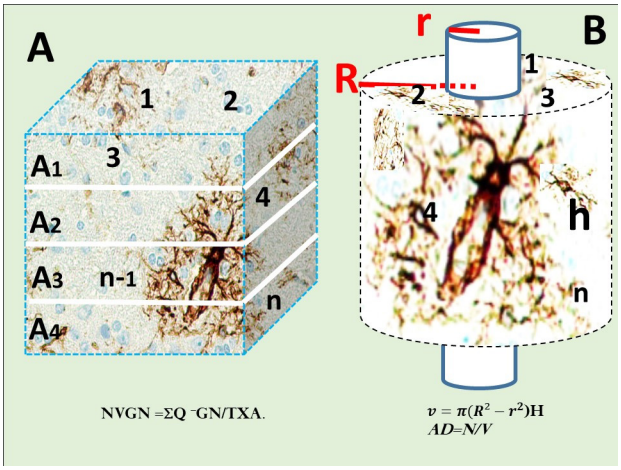

Figure 2. Neuron estimation method using a 3D cubic sample (1 µm edge) transformed from a histological BBB section divided into physical dissector pairs (A1-n) with numbered neurons. To estimate the number of neurons, neurons of each consecutive dissector pairs were calculated and multiplied with the dissector number, and the total neuron number was estimated per cubic meter. The formula used is located under the figure A/2 section. To count the number of astrocytes, arterioles accepted as cylinder and astrocytes imagined as bricked around a cylindric build. Astrocytes calculated per cubic cylinder and the formula for total astrocyte numbers are shown in the B/2 section.

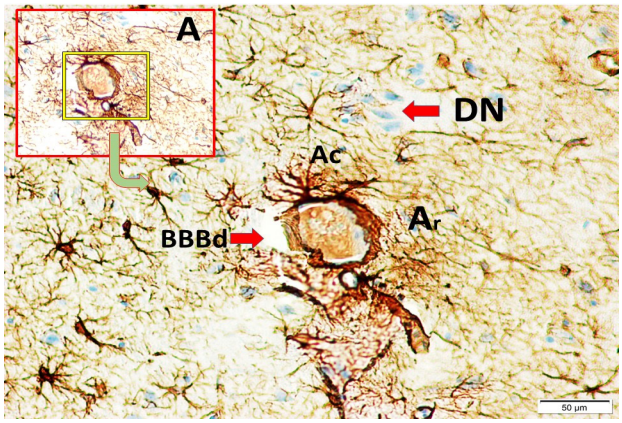

Figure 3. Histopathological appearance of partially disrupted BBB (BBBd) (LM, GFAP, ×10/A). The magnified appearance of BBBd with decreased astrocyte (Ac) numbers at the periphery of deformed cerebral arteries (Ar), astrocytes (Ac), and deformed neurons (DN) (LM, GFAP, ×40/Base) in a rat given an arrhythmogenic dose of digoxin.

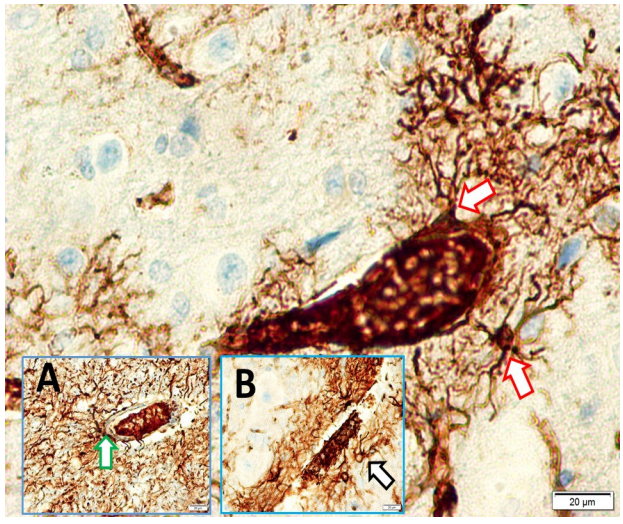

Figure 4. Histopathological appearance of a normal BBB with astrocytes (red arrows) in a normal rat (LM, GFAP, ×40/Base), partially destructed BBB, fragmented astrocytic feet (green arrow) in therapeutic dose group (LM, GFAP, ×40/A). The most destructed BBB fragmented astrocytic pedicles (black arrow) (LM, GFAP, ×40/B) in a lethal dose given rat.

Table 3: Glial/degenerated neuron ratio of BBB

Groups	Total glial cell ratio	Degenerated neuron ratio
Control	2950±513	15±4
Sham	2910±390	16±4
Therapeutic dosage	2890±215	20±6
Arrhythmogenic dosage	2360±480	218±51
Lethal dosage	1760±250	570±98

Table 4. *P* values of histopathological results

	Control/ Arrhythmogenic	Arrhythmogenic/ Lethal	Control/Lethal
P value of glial cells	0.05	0.0005	0.00001
P value of degenerated cells	0.005	0.005	0.00001

Discussion

Although the most common symptoms of digitalis toxicity are related to the cardiovascular system in adults, intestinal and neural networks are the most affected.[23] Digitalis toxicity can even cause life-threatening symptoms[24] such as fatal arrhythmia, SAH, and dangerous BBB destruction. Digitalis toxicity can include complications of the urinary, cardiovascular, respiratory, and central nervous systems. Accidental poisoning or overdose occurs most frequently in children associated with difficult feeding, vomiting, and weight loss.[5] Depression, vomiting, salivation, and anorexia are seen before ECG changes.[25] Stevens-Johnson syndrome-like findings may be observed.[26] Coronary artery disease and gastroesophageal reflux are also frequently seen following digitalis toxicity.[27] Acute digitalis overdose is characterized by high electric instability of the neural heart web.[28] Intravenous digoxin induces coronary atherosclerosis.[29]

Although cardiac glycosides are beneficial for cardiac rhythm disorders, they have adverse effects depending on the duration and dosage as well as congenital effects because they easily pass through the BBB and placenta. Digoxin should not be used in atrial fibrillation without heart failure although cardiac glycosides have been used for atrial fibrillation for 100 years.[30] Fatal cardiac arrest arising from anti-digoxin antibody production by heart tissue has been reported.[10] Besides, renal failure and hepatic disease augment digitalis toxicity[31]; the latter because digoxin elimination from the systemic circulation occurs via the bile duct.[32] Digitalis toxicity could result in intestinal dysfunction affecting the neurenteric network.[33] Because of these adverse effects, glycosides should not be used in congenital heart disease, duodenal ulcer, and gastric erosions,[34] and CNS disturbances[35] unless necessary.

Vasospasm is a significant predictor of poor clinical outcome in digoxin-induced SAH. However, digoxin might have a beneficial effect on vasospasm.[36] Marx et al declared that cardiac glycosides disrupt the BBB. Neural cell damage and neuro-necroptosis have been reported with toxic doses.[1] Autonomic nervous system toxicity, central pontine myelinolysis,[4] encephalopathies,[7] excessive vagal stimulation,[3] and fulminant hepatic failure[8] is also seen following digoxin treatment[37] and loss of effective BBB.[38]

Digoxin antibodies have vasoconstrictor activity and antihypertensive effects, and they cause intracerebroventricular or cerebral hemorrhage.[6] The most affected components are chemoreceptors and the baroreceptor network because of the denervation effect.[9] The central, autonomic, and peripheral nervous system, choroid plexus, neurohypophysis, adenohypophysis, area postrema, superior cervical sympathetic ganglion, and adrenal medulla[10] are also affected. Digoxin can affect the optic tract, optic chiasma, choroid plexus, especially of the fourth ventricle, area postrema, chemoreceptor trigger zone, and the vagal nucleus.[39] Central pontine myelinolysis is frequently seen in elderly patients with neurodegenerative disease.[4] Hippocampal injury is possible after digoxin treatment.[11] The endogenous opioid peptide-related behavior modulation may be disrupted with digoxin toxicity.[40] Digitalis toxicity may disrupt the sympathovagal control network,[41] which could lead to respiratory arrest due to vagal paralysis[42] and SAH probably due to hypothalamic damage.[12,43] Glycosides may be transported to the cerebrospinal fluid via choroidal arteries in patients with SAH.[12] The effect of digitalis toxicity one the heart resembles central sympathetic hyperactivity.[44] Digoxin plasma concentration more than 17.1 ng/mL can be a valuable diagnostic element; the therapeutic digoxin level is below 3 ng/mL.[45] Paroxysmal atrial flutter, inverted P wave, atrial tachyarrhythmia, double atrial potentials, and ventricular tachycardia are seen on the ECG due to digitalis toxicity.[46] Anti-digoxin Fab antibody fragments must be ready for all cases of digoxin toxicity presenting to the emergency department.[47]

Histological anatomy of BBB

BBB is formed by capillary endothelial cells covered with glial astrocytes and tight junctions among the endothelial cells. The tight junctions prevent dangerous particles from being transported to the brain. A normal BBB is constructed with a basal vascular membrane lined with flat endothelial and externally located pericytic extensions of astrocytes, which cover the microarterioles. The pia mater allows the entry of the blood vessels into the deep parts of the cerebral cortex. The pia mater covers meningeal vessels, forming a continuous sheet to separate the subarachnoid, subpial, and perivascular spaces. It is an effective barrier to the passage of particulate matter. The most functional parts of BBB are the luminal membrane, endothelial cells, tight junctions, and the phagocytotic astrocytes. The perivascular spaces are confluent with the only subpial space. BBB is not found in periventricular

organs such as pineal glans, subfornical organs, area postrema, subcommissural organs, eminentia medialis, and infundibulum of the neurohypophysis.

The mammalian BBB consists of endothelial cells, linked by tight junctions, and the adjacent pericytes and extracellular matrix. For example, red blood cells do not enter the perivascular spaces. If BBB is disrupted, then a large number of inflammatory cells in the subarachnoid area readily penetrate the pia mater.[48]

Histopathological findings in BBB destruction

Histomorphological, micro, and macro architectures of BBB become fragmented in all BBB pathologies. Late BBB breakdown occurs in focal head injury.[49] An edematous zone, dense homogeneous coagulation, pronounced pial arterial dilatation, and thrombus formation is characteristic histopathological features of inflamed BBB.[50] Proteinaceous materials and hematogenous cells migrate to enlarged periarteriolar capillaries in inflammatory conditions.[51] Ischemic insults result in passive dilation of cerebral vessels during cerebral edema.[52] The ischemic edema and BBB destruction occur following the first day of cerebral trauma.[53] Plasma extravasation occurs in the infarcted zone of the early few days. Proliferated and migrated endothelial cells may damage pericytic villi in the newly developed vessels because of plasma extravasation during the recovery phase.[54] Hyperthermia causes acute thermal BBB destruction in the necrotic, reactive, and permeable zones of the viable brain tissue. Damaged endothelial cells and the destruction of the tight junctions in the necrotic zone are observed.

Though numerous pinocytotic vesicles in the porous zone 6 h to 3 days after hyperthermia.[15] BBB permeability increases in brain abscess because cerebritis disrupts BBB, leading to inoculation of a suspension of bacterial fragments into the brain.[13] Purulent leptomeningitides with inflammatory cells disrupt the microcirculatory pattern of BBB.[48] BBB destruction facilitates demyelinating[16] and neurodegenerative disease development secondary to damage of BBB neural networks.[17] Thiamine deficiency results in BBB destruction-induced encephalopathy.[55] Increased corpora amylacea are observed around astrocytic processes of BBB or the cerebrospinal fluid–brain interface, along with lipofuscin accumulation and neurofibrillary tangles in all brain regions, eventually leading to neurodegenerative disease.[56] Cerebral cavernous malformations are linked to undeveloped BBB.[57]

Digitalis toxicity and BBB

Some authors acknowledged that digitalis toxicity deteriorates BBB. Intracellular ion accumulation in toxic levels, DNA fragmentation and apoptosis,[58] sympathetic inhibition related cardiopulmonary pathologies,[59] dangerous hypotension,[60] autonomic imbalances induced circulatory, respiratory, neuroendocrine abnormalities,[9] and acute lethal anaphylaxis have been reported during digoxin overdose usage. The placental transition of digoxin can be dangerous for the fetal brain[19] and autonomic imbalances[61] because of the BBB and autonomic pathways destructing effects.

Clinical importance of that study

BBB destruction can decrease neuroimmunity.[62] It is hypothesized that it might lead to neurodegenerative and tumoral pathologies of the brain after many years. Also, neuroimmunological diseases associated with inflammatory diseases.[63] The acute effects of digitalis toxicity are mostly based on acute anti-physiological blockade. In the acute stage, a precise diagnosis may not be made due to non-specific biochemical and electrophysiological changes. Since histopathological evidence cannot be collected in the early stages, the diagnosis remains challenging. If low doses of digoxin have a neurotoxic effect, it is possible that there may be BBB abnormalities such as brain stem in cardiorespiratory disturbances detected patients; or else, a high dose of digoxin toxicity could not be seen unless BBB disruption. The more destructed BBB could cause the more degenerated peri-arteriolar neurodegeneration, which results in clinical outcomes. Therefore, analytical medical history, careful physical examination, and histopathological observations is required to obtain analytical results in such toxications.

Conclusion

Biochemical and electrocardiographic findings in digoxin toxicity need to be standardized as digoxin overdose or abuse can lead to severe health and legal problems, especially in already ill children and the elderly. Digoxin should not be used in patients with multiple trauma, massive cerebropulmonary edema, bleeding diathesis, and pregnancy because all of them are considered as risk factors for the destruction of BBB.

Future Insight

The long-term effects of digoxin-induced BBB destruction and digoxin placental transport could cause defined or undefined neuroendocrinological and cardiorespiratory disabilities.

Conflict of Interest

There is no conflict of interest.

Ethical approval

Ethical approval was obtained from the Board of the Animal Experiments of Atatürk University (HADYEK-14.03.2019/44) and the study was conducted in the Experimental Animals Laboratory of Atatürk University (ATADEM).

Authors' Contribution

MNK: Methodology, validation, investigation, writing. OC: Conceptualization, methodology, investigation, data curation. DD: Investigation, methodology. KAN: Pharmacological analysis.

Study Highlights

What is current knowledge?

- Digitalis has irreversible toxicological effects on multiple organs.

What is new here?

- Higher digitalis doses were found to be linked with subarachnoid hemorrhage and blood-brain barrier (BBB) destruction.
- The more destructed BBB could cause the more degenerated peri-arteriolar neurodegeneration, which results in worse clinical outcomes.
- Digoxin toxicity induced BBB destruction and related acute/chronic neuropsychiatric complications need to be standardized as digoxin overdose or abuse that can lead to severe health and legal problems.

SO: Pathological examination. MDA: Conceptualization, supervision, writing - review & editing.

Acknowledgments

Many thanks to the pathology laboratory team

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