Original Article

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

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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-necrototic and congenital effects because they easily pass through the BBB and placenta.1 Excessive vagal stimulation-induced fatal bradycardia is a dangerous complication1 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 Encephalopathy7 and fulminant hepatic failure8 have also been reported with toxic doses.

The most affected parts of the nervous system are chemoceptors and baroreceptor networks6; 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 permeability13 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 demyelinating16 and neurodegenerative disease.17

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can increase autonomic imbalances, and cause acute lethal anaphylaxis. The placental transition of digoxin can be very dangerous for fetal brain development 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. 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 O₂ 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).

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. 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 \( P < 0.05 \), \( P < 0.005 \), \( P < 0.0005 \), and \( P < 0.0001 \).

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>
<thead>
<tr>
<th>Groups/Dosage</th>
<th>Blood Digoxin Levels</th>
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<tbody>
<tr>
<td>Control / 0 mg/mL</td>
<td>0 ng/mL</td>
</tr>
<tr>
<td>Sham / 0 mg/mL</td>
<td>0 ng/mL</td>
</tr>
<tr>
<td>Therapeutic / 0.33 mg/mL</td>
<td>1.81 ng/mL</td>
</tr>
<tr>
<td>Arrhythmogenic / 1 mg/mL</td>
<td>3070.5 ng/mL</td>
</tr>
<tr>
<td>Lethal / 1.65 mg/mL</td>
<td>4999 ng/mL</td>
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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, ×20/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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total glial cell ratio</th>
<th>Degenerated neuron ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2950 ± 513</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>Sham</td>
<td>2910 ± 390</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>Therapeutic dosage</td>
<td>2890 ± 215</td>
<td>20 ± 6</td>
</tr>
<tr>
<td>Arrhythmogenic dosage</td>
<td>2360 ± 480</td>
<td>218 ± 51</td>
</tr>
<tr>
<td>Lethal dosage</td>
<td>1760 ± 250</td>
<td>570 ± 98</td>
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Table 4. P values of histopathological results

<table>
<thead>
<tr>
<th></th>
<th>Control/Lethal</th>
<th>Arrhythmogenic/Lethal</th>
<th>Arrhythmogenic</th>
<th>Control/Lethal</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value of glial cells</td>
<td>0.05</td>
<td>0.0005</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>P value of degenerated cells</td>
<td>0.005</td>
<td>0.005</td>
<td>0.0001</td>
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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. Digitalis toxicity can even cause life-threatening symptoms 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. Depression, vomiting, salivation, and anorexia are seen before ECG changes. Stevens-Johnson syndrome-like findings may be observed. Coronary artery disease and gastroesophageal reflux are also frequently seen following digitalis toxicity. Acute digitalis overdose is characterized by high electric instability of the neural heart web. Intravenous digoxin induces coronary atherosclerosis.

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. Fatal cardiac arrest arising from anti-digoxin antibody production by heart tissue has been reported. Besides, renal failure and hepatic disease augment digitalis toxicity; the latter because digoxin elimination from the systemic circulation occurs via the bile duct. Digitalis toxicity could result in intestinal dysfunction affecting the neurenteric network. Because of these adverse effects, glycosides should not be used in congenital heart disease, duodenal ulcer, and gastric erosions, and CNS disturbances unless necessary.

Vasospasm is a significant predictor of poor clinical outcome in digoxin-induced SAH. However, digoxin might have a beneficial effect on vasospasm.

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

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 phagocytic astrocytes. The perivascular spaces are confluent with the only subpial space. BBB is not found in periventricular spaces.

declared that cardiac glycosides disrupt the BBB. Neural cell damage and neuro-necroptosis have been reported with toxic doses. Autonomic nervous system toxicity, central pontine myelinolysis, encephalopathies, excessive vagal stimulation, and fulminant hepatic failure is also seen following digoxin treatment and loss of effective BBB.

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Effect of digitalis overdose on BBB

organ systems such as pineal glans, sub fornical organs, area postrema, sub commissural organs, eminentea 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.40

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.40 An edematous zone, dense homogeneous coagulation, pronounced pial arterial dilatation, and thrombus formation is characteristic histopathological features of inflamed BBB.39 Proteinaceous materials and hematogenous cells migrate to enlarged per arteriolar capillaries in inflammatory conditions.31 Ischemic insults result in passive dilation of cerebral vessels during cerebral edema.32 The ischemic edema and BBB destruction occur following the first day of cerebral trauma.33 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.44 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 leptomeninigitides with inflammatory cells disrupt the microcirculatory pattern of BBB.41 BBB destruction facilitates demyelinating46 and neurodegenerative disease development secondary to damage of BBB neural networks.17 Thiamine deficiency results in BBB destruction-induced encephalopathy.45 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.46 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,38 sympathetic inhibition related cardiopulmonary pathologies,59 dangerous hypotension,60 autonomic imbalances induced circulatory, respiratory, neuroendocrine abnormalities,6 and acute lethal anaphylaxis have been reported during digitalis overdose usage. The placental transition of digoxin can be dangerous for the fetal brain59 and autonomic imbalances62 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 tumor 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, 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 neuroendocrine 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.
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 neuromyopathic complications need to be standardized as digoxin overdose or abuse that can lead to severe health and legal problems.

Many thanks to the pathology laboratory team for their valuable contributions.

Acknowledgments

References
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