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Research Article

DOXORUBICIN INDUCED COGNITION IMPAIRMENT IN RAT MODEL

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ABSTRACT

Objective: Doxorubicin (DOX) is a chemotherapeutic agent commonly used to treat a variety of cancers despite having well documented adverse side effects in organs like heart, liver, and kidney. The damage caused to brain, if any, and to what extent is, however, still not clear and the present study was undertaken to estimate the possible role of DOX inducing cognitive impairment in Wistar rats.

Methods: The study carried out in two groups of Wistar rats (n=6) with one group receiving DOX, and the other only normal saline used as control. During and after the experimentation period cognitive level of each rat was measured using Barnes maze (BM) till 8 weeks. At the end of the observation period (after 8th week), a series of biochemical and histopathological studies were carried out after sacrificing the animals.

Results: Errors to reach target and time taken to reach the target was found in BM experiment, elevated levels of antioxidants and hemoglobin was found reduced significantly in the DOX treated group when compared to the controls while histopathology of brain cells of DOX treated group also showed reduction in hippocampal cellularity and cell death in hippocampal area suggesting evidence of oxidative damage caused by DOX treatment.

Conclusion: Parameters in this study which not only conclusively show the damage caused to brain by DOX, but also estimates the changes caused to each indicator by this drug.

Keywords: Chemotherapy, Brain, Hippocampus, Oxidative stress.

INTRODUCTION

Chemotherapy-induced cognitive impairment is a serious challenge faced by cancer survivors. During and shortly after chemotherapy for cancer, many patients report attention deficits, memory loss, and confused thought processes [1]. It has been long known that cognitive impairment affect the quality of life and provoke feelings of depression in patients [2,3]. The fractions of patients suffering from chemotherapy-induced cognitive deficits vary widely with estimates ranging from 17-75%, based on study design and other factors [4,5]. These cognitive problems, collectively called somnolence or cognitive dysfunction, are also reported in cancer patients undergoing doxorubicin (DOX) - based chemotherapy, particularly for the treatment of breast cancer [6-8].

DOX is an anthracycline class of drug and is used as a powerful agent against a variety of cancers including breast and esophageal carcinomas; osteosarcoma, Kaposi's sarcoma, and soft tissue sarcomas [9]. Use of DOX is limited by a number of very well established side effects that include cardiotoxicity, hepatotoxicity, nephrotoxicity, etc. [10]. However, DOX-induced toxicity on brain tissue is much less understood [11]. Tangpong *et al.* 2006 showed that, DOX does not cross the BBB. However, it is hypothesized that DOX-induced circulating tumor necrosis factor (TNF) lead to mitochondrial dysfunction in the brain which might be responsible for the cognitive disorder.

The present study was carried out to understand the extent of damage, if any, caused to the brain by DOX treatment in Wistar rats.

METHODS

Animals

The study was carried out after obtaining approval from Institutional Animal Ethical Committee of RMRC (CPCSEA No. 1388/c/10/CPCSEA). Albino rats (Wistar strain) weighing between 150 and 250 g, procured from M/s Venkateshwara Enterprises, Bengaluru, were used in the experiment. Prior to initiation of the experiments, rats were acclimatized for 1 week in laboratory conditions. Drinking water and standard rat feed was provided during this time.

Materials

Commercial DOX injection (DOXUTEC 10; United Biotech (P) Ltd. India) was used in the study.

Barnes assay

Barnes maze (BM), a sensitive standard tool for testing hippocampus-dependent spatial memory in rats [12,13] was used in the study for estimation of cognition levels of rats.

Experimentation

After acclimatization, rats were divided into two groups, each consisting of six rats. Group I served as control, where only saline solution was administered by intraperitoneal route (5 ml/kg body weight) while Group II was DOX treated group where DOX was injected intraperitonially after dissolving in saline (4 mg/kg body weight) weekly once for 4 weeks. Rats were observed for 3 weeks for their behavior and mortality.

Learning and memory test in rats were performed by using BM.

On day 1, rats were familiarized on BM to make them comfortable. Day 2-8 was a training period for rats on the BM. Experiment was started after acclimatization period of 8 days. Experimental observation was recorded daily before dosing period, during dosage period, and after dosing period.

Rat blood samples were collected from eye orbit under halothane anesthesia on day 1 before treatment and 7th week after treatment with DOX.

After the dosing period and observation period of 3 weeks for control and DOX groups respectively, rats were humanely sacrificed under halothane anesthesia and their brain dissected out to perform antioxidant study and estimate histopathological changes, if any.

Brain tissue was cleaned in ice-cold saline and weighed immediately. Brain tissue from each group was taken and 30 homogenate was prepared in saline solution buffered with KCl (pH 7.4). Each sample was subjected to estimation of glutathione (GSH) [14], lipid peroxidation in terms of thiobarbituric acid reactive species, using malondialdehyde as standard by the method of Buege and Aust., superoxide dismutase (SOD) [15] and catalase (CAT) [16]. Brain samples of at least two animals from each group were fixed in 10% formalin solution for histopathological study.

Statistics

Statistical data was expressed as mean ± standard deviation or mean ± standard error mean (n=6). Statistical comparisons were performed using Student's t-test by using GraphPad Software, instat3. $p<0.05$ was considered statistically significant.

RESULTS

Behavior study

After exposing the Group II (DOX treated) rats to the BM task, they showed initial freezing behavior followed by grooming and this behavior extended for more than 5 minutes every time during the task.

Errors to reach target and time taken to reach the target

Before drug administration, the rats were trained and the errors were noted.

During the dosing period

During dosage period, time taken to reach the hole and number of errors in attempts to reach the target hole in BM in the DOX treated group were counted and compared with that of control. Number of errors to reach the target and time taken to reach the target was increased in DOX group in comparison to rats treated with normal saline (Tables 1 and 2).

After dose administration: After dosing period rats were kept under observation for 3 weeks.

Antioxidant activity

Brain tissue analysis indicated that there was an increase in free radical generation and antioxidant defense was impaired in DOX induced group. Antioxidant assay for GSH, lipid peroxidation, SOD, and CAT from brain tissue is shown in Table 3. There was a significant decrease in brain GSH, CAT, and SOD levels in DOX induced group. However, lipid peroxidation level increased in DOX induced group.

Hemoglobin level

A significant reduction in hemoglobin level was noted in rats of the DOX treated group when compared with the control group (Graph 1).

Histopathology

In sharp deviation to normal histopathology of brain as seen in the control animals, the samples from the DOX treated group showed neuronal eosinophilia, neuronal nuclear pyknosis, neuronal karyorrhexis, astrocytic changes, macrophage influx, hippocampal cellularity, and hippocampal cell death (Table 4).

DISCUSSION

Previous reports of preclinical studies have shown that several commonly used chemotherapeutic agents can induce central oxidative stress in healthy rodents [17,18]. The results of our study corroborate this fact with the specific use of DOX as the drug. BM experiments revealed that the number of errors and time taken to reach the target hole is significantly more in DOX treated group, indicating detrimental effect on spatial reference learning, and memory with depressed irritating behavior in DOX treated rats when compared to normal.

Table 1: Number of errors to reach the target hole

Experimental Days	Control	DOX
Before dosing		
Day 1	14.500±3.271	16.667±6.022
Day 2	13.000±2.280	13.333±3.933
Day 3	9.500±1.049	7.333±3.077
Day 4	2.333±2.066	1.667±1.366
During dosage period		
Day 5	2.667±1.366	1.667±1.033
Day 6	2.167±0.7528	2.333±1.633ns
Day 7	2.333±0.5164	3.000±1.414[a]ns
Day 8	2.500±1.378	4.500±1.378[a]ns
After dosing		
Day 9	2.333±1.211	5.333±1.506[a]ns
Day 10	1.000±0.8944	5.667±1.633[a]***
Day 11	1.000±0.8944	5.667±0.8165[a]***
Day 12	1.333±0.5164	7.000±1.673[a]***

Values are expressed in mean±SD for n=6, Significant difference as compared to DOX followed by Student's *t*-test, control compared with DOX *p<0.05, **p<0.01, ***p<0.001 considered significant. DOX: Doxorubicin, SD: Standard deviation, ns: Non-significant, [a]: Control

Table 2: Time taken to reach the hole

Experimental Days	Time taken to reach hole in seconds (S)	
	Control	DOX
Before drug administration		
Day 1	144.17±35.606	207.33±23.321[a]**
Day 2	96.667±7.312	184.67±16.884[a]***
Day 3	68.500±11.675	98.000±7.642
Day 4	50.333±9.374	87.167±6.969
During dosage period		
Day 5	35.500±10.114	92.000±4.427[a]***
Day 6	32.667±7.474	99.000±6.782
Day 7	31.167±8.495	108.50±7.503***
Day 8	26.667±7.367	109.33±7.891***
After dosing		
Day 9	25.500±6.285	126.67±8.287
Day 10	21.667±7.711	151.00±14.913[a]***
Day 11	20.333±6.947	170.00±7.563[a]***
Day 12	19.500±6.091	190.83±9.847[a]***

Values are expressed in mean±SD for n=6. Significant difference as compared to DOX followed by Student's t-test, control compared with DOX. *p<0.05, **p<0.01, ***p<0.001 considered significant. DOX: Doxorubicin, SD: Standard deviation. [a]: Control

Behavioral models became particularly useful for the neurobiological mechanisms of behavioral perseverations and stereotypes. According to Seeger *et al.* 2004 [19], impairment in set-shifting in the Barnes circular maze task, significantly more attempts and longer time in returning to the starting hole, by the test animal, implicates increased perseverative behavior, which is also referred to in the literature as behavioral inflexibility. Recent findings suggested that this characteristic reduction in behavioral flexibility may be associated with abnormal function of the prefrontal cortex and basal ganglia [19,20], and possibly in the hippocampus [19]. In the present study, we found that there is excessive grooming, freezing, vocalization, and perseverative behavior while performing BM task within the DOX treated group of rats. It has been earlier stated that, freezing behavior is characterized by changes in blood pressure and lengths of time in crouching position, but it also is known to cause changes such as shortness of breath, increased heart rate, sweating, or choking sensation [21].

It has been postulated that DOX does not cross the blood brain barrier [22,23], however, it is understood that the circulating levels of TNF can pass through blood brain barrier and induce neuronal damage [24,25]. The cytotoxicity of TNF depends on induction of mitochondrial permeability transition pore [26]. Thus, it is possible

Table 3:Antioxidant activity of brain tissue

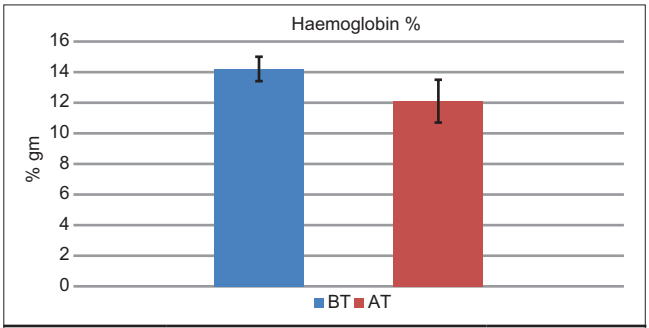
Experimental groups	Glutathione (nmol/g of wet tissue)	Lipid peroxidase (nmol/g of wet tissue)	SOD (units/mg of protein)†	CAT (units/mg of protein)††
Control	12.815±1.563	17.530±0.7243	33.530±0.5794	81.798±0.3240
DOX	9.602±0.6490[a]***	22.117±0.8119[a]***	24.957±6.504[a]*	77.703±2.161[a]**

Values are expressed in mean±SD for n=6, significant difference as compared to DOX followed by Student's t-test, control compared with DOX. *p<0.05, **p<0.01, ***p<0.001 considered significant. †One unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NTB reduction in 1 minutes, ††One unit of H_2O_2 consumed/minute. DOX: Doxorubicin, SD: Standard deviation. SOD: Superoxide dismutase, CAT: Catalase, [a]: Control

Table 4: Histopathological changes in brain

Microscopy	Control	DOX
Cerebral edema	-	++
Cerebral congestion	+	++
Meningeal congestion	-	+
Neuronal microvacuolization	-	-
Neuronal eosinophilia	-	+
Neuronal nuclear pyknosis	-	+
Neuronal karyorrhexis	-	+
Astrocytic changes	-	+
Neutrophilic infiltration	-	-
RBC extravasations	-	-
Macrophage influx	-	+
Vascular proliferation	-	-
Reactive gliosis	-	-
Demyelination	-	-
Hippocampal cellularity	Normal	Reduced
Hippocampal cell death	-	+

-: Nil, +: Mild, ++: Moderate, RBC: Red blood cell, DOX: Doxorubicin



DOX treatment	% Hemoglobin
BT	14.2±0.76
AT	12.1±1.34*

Values are expressed in mean±SD for n=6, significant difference as compared to DOX followed by Student's t-test, control compared with DOX. *p<0.05 considered significant. BT: Before treatment, AT: After treatment, DOX: Doxorubicin, SD: Standard deviation

Graph 1: Percentage of hemoglobin change in before and after treatment of doxorubicin

that the increase in TNF levels may be related to DOX induced oxidative stress and morphological injury. Free radicals play an important role in the genesis of structural and functional changes of neuronal membrane that could be responsible for the beginning or aggravation of the basic disease [27-29]. Decrease in levels of GSH can cause oxidative stress that lead to an increase in lipid peroxidation [30]. This increased lipid peroxidation level in brain results in the process of cell damage [31]. GSH acts as protective agent against cellular free radical-mediated oxidative damage by functioning as an oxyradical scavenger, thereby reducing lipid peroxidation [32-35].

Joshi *et al.* in 2005 reported a significant increase in levels of protein oxidation and lipid peroxidation in brain tissues after 72 hrs of a single i.p. injection of DOX [36]. In the present study, it was observed that there was a significant increase in lipid peroxidation and decrease in GSH, SOD, and CAT enzymes which are most likely responsible for further damage of brain tissue.

Relationship with hemoglobin and cognition is not clear. However, in the present study, DOX treatment has been found to decrease hemoglobin levels in rats. Vearncombe reported that reduced hemoglobin levels, together with increased anxiety, predict a decline on measures of cognitive function [37].

In the present study, pathophysiological investigations revealed that DOX treated rats showed a decrease in hippocampal cellularity and increase in hippocampal cell death. According to Seigers *et al.* 2008 [38], hippocampal cell death is directly proportional to the cognitive impairment in rats. Furthermore, in the present study we also observed cerebral edema, meningeal congestion, neuronal eosinophilia, neuronal nuclear pyknosis, astrocytic changes, and macrophage influx in DOX treated rats that were not observed in the control group, which may indicate impact on memory of rats.

CONCLUSION

The present study estimates changes to possible indicators of neurotoxicity in animals treated with DOX in comparison to normal control. To the best of our knowledge, this is the first study using maximum number of parameters which not only conclusively show the damage caused to brain by DOX but also estimates the changes caused to each indicator by this drug. The finding that DOX generated imbalance in oxidative stress and antioxidant enzymes in brain may have damaged the normal cellular structure in brain hippocampal area that is responsible for cognition impairment should help investigators in their quest for suitable antidote/protective agent for brain damage

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Ethics approval

The study was approved by Institutional Ethics Committee.

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