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Evaluation of Doxorubicin for its Chemobrain-Inducing Potential in Female Rats

6.1. Introduction
DOX is one of the most widely used broad spectrum anticancer agents for almost all kinds of cancers, viz., various types of leukemia, cervical, ovarian cancers. DOX is the drug of choice till date, for the treatment of breast cancer either alone or in combination with other cytotoxic agents like, cyclophosphamide, 5-fluorouracil and methotrexate etc. However, when we use a combination of agents to model chemobrain preclinically, the individual impact or influence of cytotoxic agents cannot be explored which would actually enables oncologists for risk/benefit analysis. Hence we evaluated the influence of a single cytotoxic agent, i.e. DOX in inducing chemobrain associated cognitive complications. Despite of its use globally, not much attention has been paid for its neurotoxicity and the associated neurological complications including chemobrain.

Chemobrain studies investigating cognitive complications in animal models are scarce and the existing studies have used mainly male animals in which DOX treatment was given on a single high dose basis which is not clinically relevant (Aluise et al., 2010). At present, literature supporting DOX influence on cognitive functioning is mixed and ambiguous. Hence, we have selected DOX to evaluate its chemobrain-inducing potential in female rats on multiple treatment which is relevant to clinical scenario.

6.2. Materials and Methods

6.2.1. Animals
Female Wistar rats of body weight ranging from 180-200 g were procured from CARF of Manipal University, Manipal. All the experimental procedures were approved by IAEC with approval number, IAEC/KMC/17/2013.

6.2.2. Chemicals and apparatus
DOX (Fresenius Kabi, Solan), normal saline were procured and apparatus was similar to that mentioned in chapter 4, page 24.

6.2.3. Experimental design
Initially during the pilot studies to fix the chemobrain-inducing schedule with DOX, it was given at 2.5 and 3.5 mg/kg by intraperitoneal and intravenous routes at a dose volume of 2 ml/kg in sample animals (n=3) to test the tolerability and for any other toxic symptoms. The dosing was continued once in 5 days upto 10 cycles and the daily observations were made for any mortality or morbidity.
Chapter 6  Evaluation of DOX for its Chemobrain-Inducing Potential

We found that, female rats were able to tolerate DOX at 2.5 mg/kg, i.p., once every 5 days upto 50 days (10 treatment cycles) without any co-morbidity or the mortality. Intravenous administration of DOX resulted in severe erosion and inflammation resulting in swelling of tail vein with severe degeneration of cartilage superficially. This led to the cessation of dosing after 4 cycles of DOX treatment. Furthermore, mortality and morbidity was noticed in intravenous DOX treated animals at shorter duration of treatment, i.e. after 4 cycles of DOX. Hence we confirmed that, intraperitoneal administration of DOX would be ideal for studying chemobrain induction rather than intravenous route. And moreover, if morbidity is present, it will become confounding influence which can affect the cognitive assessment.

Two treatment groups (n=12 for individual group) were used in each model for assessing either episodic or spatial memory processes using novel object recognition and Morris water maze task respectively. One group of rats were treated with normal saline (0.9 % w/v), i.p. while the second group was treated with DOX, i.p. Either DOX at 2.5 mg/kg, i.p. or normal saline, i.p. was administered once in 5 days upto about 10 cycles to the individual experimental groups of animals at a constant dose volume of 2 ml/kg. Following the completion of dosing schedule, rats were subjected to behavioural analysis to assess episodic or spatial memory. Scheme of the protocol was shown in Fig. 6.1 below.

Fig. 6.1. Schematic diagram of protocol followed for inducing chemobrain by DOX

6.2.4. Apparatus

Apparatus used for assessing episodic memory was same as that mentioned in chapter 4, page 24. For evaluating the spatial memory, we have used Morris Water Maze (MWM) by using the ANYmaze apparatus. MWM apparatus comprises of a black circular pool tank (having diameter 120 cm) filled with water which was maintained at a constant temperature of 25 ± 2º C. It was arbitrarily divided into 4 hypothetical quadrants (Q1 to Q4) and a circular platform of 10 cm in diameter was placed in one of the four hypothetical quadrants (Q4). The maze was filled with water upto 1 cm above the platform. A visual cue was provided which served
as spatial cue and assisted in finding the hidden platform. The position and location of spatial cue and platform were maintained constant throughout the acquisition trials. The behavioural assessment of animals was monitored using ANY-maze video tracking system (Software version 4.99m, Stoelting Co., San Diego Instruments, USA).

6.2.5. Object recognition task (ORT)
ORT was conducted as mentioned in chapter 4, page 24 and 25 using an ITI of 2 h.

6.2.6. Morris water maze (MWM) task
Spatial working memory processing requires functional integration of hippocampus and the associated structures which are part of limbic system in CNS. We have used Morris water maze paradigm to assess this spatial working memory in female rats as we noticed in ORT that, female rats can be used to assess the cognitive processing without any estrus cycle interference.

Spatial memory is generally assessed using Morris water maze and standard procedures were followed according to the earlier studies with some modifications (Morris, 1984). The protocol comprises of 4 days of acquisition trials for assessing acquisition learning followed by a retention/probe trial on 5th day for assessing retention/recall memory. During the acquisition trials from day 1 to day 4, individual rats received four trials per day and were allowed to find hidden platform by using a visual cue. Latency to reach the hidden platform (escape latency) and path length were analysed using ANYmaze video tracking system.

During retention trial, each rat was subjected to one trial without placing platform over a period of 60 sec and latency to reach the target quadrant (Q4) i.e. the quadrant having platform during acquisition trials as well as the time spent in the target quadrant were noted using the any maze software and compared.

6.2.7. Change in body weight
Body weight was monitored once in every 3 days and the average body weight was compared among the two groups.

6.2.8. Hematological Profiling
Complete blood profiling was carried out as discussed in chapter 5, page 34.

6.2.9. Open field test
Distance traveled by the rats along with mean speed during a 15 min trial duration was measured using ANYmaze software (Masur et al., 1980; Prut and Belzung, 2003).
6.2.10. Statistical analysis

Target latency and path length during acquisition trials, target quadrant (Q4) latency or time spent in target quadrant in retention trial as well as the data for hematology, locomotion, acquisition as well as retention memory are analysed by Students’ unpaired t-test.

6.3. Results

6.3.1. Episodic memory in ORT

![Graphs showing exploration time, discriminative index, and recognition index for familiarization and recognition trials for saline control and DOX control groups.](image)

**Fig. 6.2.** Effect of DOX on episodic memory in female rats. Data represents mean ± SEM of (a) and (b) Exploration time in familiarization or recognition trial respectively; (c) and (d) Discriminative and Recognition indices respectively. ***p<0.001 vs. familiar object, ***p<0.001 vs. saline control (n=11-12).

It was found that discriminative and recognition indices were significantly (p<0.001) reduced for the group treated with chronic DOX when compared to saline control. Also animals of DOX group spent almost equal time exploring both novel and familiar objects in recognition trial which indicates that they were not able to discriminate the novel object from the familiar one. This shows that chronic DOX treatment has resulted in episodic memory deficits in Wistar rats indicative of chemofog like condition (Fig. 6.2)

6.3.2. Spatial memory in MWM

Significant differences were not observed for the acquisition learning parameters tested viz., target latency and path length from day 1 to day 4 between saline control and DOX treated group. Also there was no difference among the groups for the probe trial measures such as
Q4 latency and Q4 time which indicates that DOX did not affect acquisition learning or recall memory (Fig. 6.3).

**Fig. 6.3.** Effect of DOX on spatial learning and memory in female rats. Data represents mean ± SEM of (a) Target latency and (b) Path length during acquisition learning; (c) Q4 Latency and (d) Q4 Time during retention trials (n=12).

### 6.3.3. Haematological analysis

DOX treatment resulted in myelosuppressive effects with reduction in RBC, haemoglobin levels along with a comparative reduction in leukocytes when compared to control group (Table 6.1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>Saline control</th>
<th>DOX control</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (×10⁶ cells/µl)</td>
<td></td>
<td>8.24 ± 0.166</td>
<td>5.23 ± 0.319*</td>
</tr>
<tr>
<td>WBC (×10³ cells/µl)</td>
<td></td>
<td>8.89 ± 0.80</td>
<td>6.61 ± 0.48</td>
</tr>
<tr>
<td>Hb % (g/dl)</td>
<td></td>
<td>13.86 ± 0.122</td>
<td>9.81 ± 0.158 *</td>
</tr>
<tr>
<td>Lymphocytes (×10³ cells/µl)</td>
<td></td>
<td>6.04 ± 0.949</td>
<td>5.42 ± 0.455</td>
</tr>
<tr>
<td>Monocytes (×10³ cells/µl)</td>
<td></td>
<td>1.21 ±0.103</td>
<td>1.12 ±0.05</td>
</tr>
<tr>
<td>Platelets (×10³ cells/µl)</td>
<td></td>
<td>638 ± 23.545</td>
<td>570 ± 22.497</td>
</tr>
</tbody>
</table>

**Notes:** Data represents mean ± SEM of various blood parameters, *p<0.001 vs. saline control (n=6).
6.3.4. Locomotor activity in open field test

Locomotor activity of the animals treated with 10 cycles of DOX was not hindered as compared to the saline controls which was evident from the insignificant difference in locomotor activity parameters, i.e. mean speed and distance traveled among saline control and DOX control groups. This indicates that treatment with DOX did not influence the locomotor activity of the animals. Hence it is appropriate to assess the cognitive function in these animals without any confounding influences with DOX dosing schedule followed (Table 6.2).

Table 6.2. Effect of DOX on locomotor activity in open field test.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Treatment</th>
<th>Distance (cm)</th>
<th>Mean velocity (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline control (CMC, 2 ml/kg, p.o.)</td>
<td>4650 ± 24.5</td>
<td>7.2 ± 0.85</td>
</tr>
<tr>
<td>2</td>
<td>DOX control (2.5 mg/kg, i.p.)</td>
<td>4707 ± 37.6</td>
<td>7.6 ± 0.13</td>
</tr>
</tbody>
</table>

Notes: Data represents mean ± SEM of distance traveled and mean velocity. No significant differences was observed among saline control and DOX control groups (n=6).

6.3.5. Change in body weight

It was found that, the average body weight of the group treated with DOX was comparatively low with respect to saline control at the end of the study period. However, this difference was not statistically significant (Data not shown).

6.4. Discussion

Chemotherapeutic agents can target normal healthy cell population including neurons owing to their nonspecific action on brain which may possibly underlie the chemobrain condition. It is essential to find the cytotoxic agents given as a part of chemotherapy for their potential impact on producing chemobrain associated cognitive dysfunction for diverse components of memory.

Therefore in the present study, we have tested DOX, the most widely used broad spectrum anthracycline anticancer antibiotic for many solid and liquid cancers, for its chemobrain-inducing potential on episodic and spatial components of cognitive process. Further, not much attention has been paid on DOX induced neurotoxicity and its role in inducing cognitive impairment despite of its global use for many forms of cancers.

Although DOX cross BBB to a lesser extent, the reported chemobrain complications are high with DOX based chemotherapy in BC survivors. Some of the earlier reports also revealed
that DOX can cause neurotoxicity through an indirect, i.e. peripheral inflammatory (TNF-alpha and NF-kB) mediated neurotoxic mechanisms (Aluise et al., 2010; Thorn et al., 2011; Watkins et al., 1995). Scientists revealed that DOX impairs cognitive function by inhibiting the hippocampal neurogenesis in male rats following 4 weekly doses (Christie et al., 2012). Further, DOX treatment has been reported to induce central neurotoxicity in mice through generation of inflammatory markers and lipid/protein oxidation products (Tangpong et al., 2011).

We have validated and followed a predetermined dosing schedule (based on the pilot studies in our laboratory) for inducing chemobrain like condition with DOX. DOX was given over a period of 50 days with fixed dose and dosing interval. In our study, we noted that 10 cycles of DOX has resulted in significant deficits of episodic memory without any mortality. However, the spatial memory component was found to be unaffected.

In ORT study, we found that vehicle treated rats were able to discriminate the novel object from the familiar one at an ITI of 2 h whereas, rats treated with DOX were not able to recognize novel object and were not discriminating the novel object from the familiar one. Also it was found that, recognition and discriminative indices were significantly reduced for the group treated with chronic DOX as compared to saline control. This indicates that chronic DOX treatment has produced episodic like memory deficits in female Wistar rats, the condition which may correlate with cognitive complications observed in chemobrain condition in cancer survivors.

In MWM study, we observed that rats treated with either saline or DOX performed equally well in identifying the hidden platform during acquisition learning. Also the Q4 latency and Q4 time during the probe trial were not significantly different for saline and DOX treated groups. This infers that chronic DOX treatment did not affect the spatial learning as well as spatial retention memory. Further, a previous report showed that treatment with chemotherapeutic agents, 5-fluorouracil and cyclophosphamide resulted in transient improvement of spatial learning in MWM task despite of its toxic potential on hippocampus (Lee et al., 2006). In the present study also, DOX did not impair spatial memory which support the above earlier finding. Further, DOX did not impair passive avoidance learning in mice as noticed in a previous study (Sieklucka-Dziuba et al., 1998).

It was also noted that, DOX has produced myelosuppression in brain after 10 cycles which could be a possible reason for episodic memory deficits observed in the present study. Different anatomical structures are involved in processing of diverse forms of memory. Spatial acquisition memory requires normal and healthy hippocampal functional integrity.
(Morris et al., 1982), while episodic memory requires major contribution from the frontal lobe cognitive processing although hippocampal functioning is also involved (Fletcher et al., 1998). This may be the fact behind the differential effects of DOX on episodic and spatial memory. Locomotor activity was not affected by DOX treatment as we found no difference in the distance traveled, mean speed among the treatment groups. This indicates that DOX treatment did not influence the cognitive assessment in either NORT or MWM tasks for episodic or spatial learning respectively.

Adult hippocampal neurogenesis (AHN) is a continuous process throughout the life span and is essential to form the new recognition memories. 700 new neurons will be added to each hippocampi daily (Spalding et al., 2013). Even when a small quantity of cytotoxic drugs enter the brain, it is highly toxic to this AHN pool of neuronal stem cells. This may be a possible reason for the deficits observed in the present study as a result of chronic DOX treatment.

6.5. Conclusion

DOX on chronic administration has produced chemobrain like condition with impaired episodic memory, however the spatial memory was not affected with DOX dosing schedule followed. Hence we conclude that DOX affects specific forms of memory without influencing locomotion and other memory components like spatial memory.
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Bibliography


