Contents

Evaluation of Doxorubicin for its Chemobrain-Inducing Potential in Mammary Cancer Rats ................................................................. 89

10.1. Introduction ........................................................................................................... 89
10.2. Objectives ........................................................................................................... 90
10.3. Materials and Methods ....................................................................................... 90
10.3.1. Animals ........................................................................................................... 90
10.3.2. Chemicals ....................................................................................................... 90
10.3.3. Apparatus ...................................................................................................... 90
10.3.4. Experimental design ...................................................................................... 90
10.3.5. Formulations and treatments ....................................................................... 90
10.3.6. Object Recognition Task (ORT) ................................................................. 90
10.3.7. Morris Water Maze (MWM) Task ............................................................... 91
10.3.8. Change in body weight .................................................................................. 91
10.3.9. Hematological Profiling ............................................................................... 91
10.3.10. Open field test ............................................................................................ 91
10.3.11. Tumor profiling .......................................................................................... 91
10.3.12. Statistical analysis ...................................................................................... 91
10.4. Results ............................................................................................................... 91
10.4.1. Object Recognition Task (ORT) ................................................................. 91
10.4.2. Morris Water Maze (MWM) Task ............................................................... 92
10.4.3. Change in body weight .................................................................................. 93
10.4.4. Haematological Profiling ............................................................................. 94
10.4.5. Open field test ............................................................................................. 94
10.5. Discussion ........................................................................................................... 96
10.6. Conclusion ......................................................................................................... 97
Evaluation of Doxorubicin for its Chemobrain-Inducing Potential in Mammary Cancer Rats

10.1. Introduction
Almost all of the earlier animal studies exploring chemofog-inducing nature of cytotoxic drugs have made use of healthy normal animals to exclude the interfering effects of immune components. And many of the investigations have employed acute and high doses of chemotherapeutic agents in rats and mice to study the behavioural dysfunction. Because of high variation in the type of chemotherapy employed, use of diverse strains and species of animals, different chemotherapy dosing schedules, the existing literature reveals that the results are mixed and ambiguous with the resulting inconclusive outcomes.

However there exists an unmet need to develop most relevant animal models to produce reproducible results and ultimately with a reliable experimental outcome. This could be achieved by developing neurocognitive animals models for simple forms of memory in cancer animal models (following chemotherapy), especially the mammary carcinoma model with a possible clinical relevance to the breast cancer surviving populations.

It is not feasible to study the individual cytotoxic drugs in humans for their chemobrain-inducing effect as clinically oncologists most often, uses a combination of chemotherapeutic drugs to treat various neoplastic disease conditions.

Moreover, clinically most of the patient related and disease related factors cannot be controlled which can affect the disease and study outcome. Hence the animal models are the precious tools which will allow the experimenter to control various factors related to age, gender, disease stage, type, duration and frequency of treatment etc.

In the previous chapters, it was found that in healthy female rats, DOX treatment for 10 cycles (at 2.5 mg/kg, i.p.) over 50 days has resulted in episodic memory deficits in object recognition task, whereas spatial memory was unaffected.

Hence, in this present chapter, we focused on evaluating the behavioural dysfunction, i.e. chemobrain-inducing potential of DOX in mammary carcinoma bearing rats so as to identify the most relevant preclinical model for exploring chemobrain complications associated with DOX chemotherapy.

Following 10 cycles of DOX chemotherapy, mammary cancer animals were subjected to behavioural testing. Further, we have evaluated locomotion and the cancer related parameters like, tumor volume, hematological profiling etc.
Chapter 10  Evaluation of DOX for Chemobrain in Mammary Cancer Rats

10.2. Objectives

Primary objective
To evaluate the chemobrain associated cognitive dysfunction inducing potential of DOX on a long term treatment basis in mammary carcinoma bearing rats

Secondary objective
To evaluate the antitumor activity of DOX in preventing the mammary carcinoma and profiling of tumor parameters

10.3. Materials and Methods

10.3.1. Animals
Twenty four female mammary carcinoma bearing SD rats of age, 15-20 weeks were used in this study. All the experimental procedures were approved by IAEC with approval number, IAEC/KMC/17/2013.

10.3.2. Chemicals
NMU (Sigma-Aldrich, NY, USA), DOX (Fresenius Kabi, Solan, HP, India), Dipotassium EDTA etc. All the other chemicals used in this present study were of reagent grade.

10.3.3. Apparatus
Apparatus used was similar to that used in previous chapters for ORT and MWM tasks.

10.3.4. Experimental design
Two experimental groups which are age and tumor volume controlled were used in this study. Group 1 was mammary carcinoma control group and second group was for evaluating the effect of DOX in mammary carcinoma condition. Following the completion of dosing schedule, animals were subjected to behavioural assessment in ORT & MWM tasks to assess the effect of DOX in mammary carcinoma rats. At the end of the study period, tumor profiling was carried out for tumor volume, hematology etc.

10.3.5. Formulations and treatments
DOX was prepared in normal saline at a dose volume of 2 ml/kg from a stock solution of 2 mg/ml. DOX was prepared prior to the administration.

Following the grouping of mammary carcinoma bearing animals, the tumor control group was treated with saline whereas group-2 was treated with DOX (2.5 mg/kg, i.p.) every 5 days at a dose volume of 2 ml/kg up to 50 days.

10.3.6. Object Recognition Task (ORT)
The basic procedure involved was same as that used in previous chapters. An ITI of 2 h was used in cancer bearing animals also during object recognition task. Time spent by the rats exploring individual objects either in familiarization or choice trial was noted and compared
between the objects and within the group by Student’s paired $t$-test. Discriminative and recognition indices were also calculated and compared among the treatment groups by unpaired Student’s $t$-test.

**10.3.7. Morris Water Maze (MWM) Task**

The methodology followed was same as that used in chapter 6, page 47.

**10.3.8. Change in body weight**

Body weight recordings were monitored weekly once throughout the study period and average body weight was analysed and reported.

**10.3.9. Hematological Profiling**

Haematological profile was assessed using blood cell counter as mentioned on page 57.

**10.3.10. Open field test**

Distance travelled and mean velocity were assessed using ANY-maze video tracking system.

**10.3.11. Tumor profiling**

Tumor volume was monitored initially before starting of the dosing schedule and at the end of the study period. Animals were also monitored in case of any morbidity or mortality and the same was recorded and reported.

**10.3.12. Statistical analysis**

Recognition and discriminative indices were analysed by Student’s unpaired $t$-test among the two test groups. MWM data, i.e. target latency, path length, swim speed and Q4 latency & Q4 time were analysed by Student’s unpaired $t$-test among the groups. Data for open filed test, body weight changes and tumor volumes were also analysed by Student’s unpaired $t$-test.

**10.4. Results**

**10.4.1. Object Recognition Task (ORT)**

It was found that, mammary cancer control group could remember the familiar object, hence this group was able to discriminate between the objects after an inter trial interval (ITI). This infers that, mammary cancer do not have any negative effect by itself on episodic memory processing for recognition of familiar objects as we noticed in chapter 9. However, mammary cancer animals upon long-term treatment with DOX for 10 cycles could not discriminate between the familiar and novel objects following an ITI of 2 h which indicates that, these animals were not able to remember the familiar object, hence they did not identify the novel object from the familiar one. Furthermore, a significant reduction in RI & DI was noted for the group treated with DOX as compared to tumor control group (Fig.10.1).
Fig. 10.1. Effect of DOX on episodic memory in ORT. Data represents mean ± SEM of (a) Exploration time during recognition trial, ***p<0.001 vs. familiar object, (b) Recognition index, (c) Discriminative index among two test groups. **p<0.01, ***p<0.001 vs. tumor control (n=12).

10.4.2. Morris Water Maze (MWM) Task
Mammary carcinoma control group was able to identify the platform over four days of acquisition trials using spatial cue whereas, DOX treated mammary cancer animals were not able to identify the location of platform using spatial cue provided.
Furthermore retention memory during probe trial was not influenced by mammary carcinoma per se. However the long term treatment with DOX resulted in deficits of spatial retention memory as well. This confirms that, mammary cancer per se do not have any negative effect on either spatial working memory or retention memory whereas DOX has negative effect on spatial working memory and retention memory during acquisition and probe trials respectively (Table 10.1 & Fig. 10.2).
Table. 10.1. Effect of DOX on spatial acquisition learning in mammary carcinoma bearing animals during acquisition trials in MWM test.

<table>
<thead>
<tr>
<th>Day</th>
<th>Group</th>
<th>Target latency (s)</th>
<th>Path length (m)</th>
<th>Swim speed (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tumor control</td>
<td>54.34 ± 3.12</td>
<td>6.61 ± 0.12</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>DOX control</td>
<td>58.84 ± 2.34</td>
<td>8.24 ± 0.78</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>Tumor control</td>
<td>50.65 ± 3.66</td>
<td>5.02 ± 0.66</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>DOX control</td>
<td>57.69 ± 5.11 *</td>
<td>7.28 ± 0.19 *</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>Tumor control</td>
<td>42.15 ± 4.19</td>
<td>4.88 ± 0.77</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>DOX control</td>
<td>51.33 ± 4.65 **</td>
<td>7.11 ± 0.91 **</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>Tumor control</td>
<td>38.66 ± 6.35</td>
<td>3.80 ± 0.89</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>DOX control</td>
<td>49.55 ± 3.43 **</td>
<td>6.97 ± 0.57 **</td>
<td>0.16 ± 0.01</td>
</tr>
</tbody>
</table>

Notes: Data represents mean ± SEM of escape or target latency, path length and swim speed, *p<0.05, **p<0.01 vs. tumor control (n=12).

Fig. 10.2. Effect of DOX on spatial retention memory in mammary carcinoma bearing animals during probe trial in MWM test. Data represents mean ± SEM of (a) Time spent in target quadrant (Q4 time) and (b) Time taken to first entry of the target quadrant (Q4 latency), **p<0.01 vs. tumor control (n=12).

10.4.3. Change in body weight

There was a relative decrease in the body weight of mammary carcinoma control animals as compared to the DOX treated mammary carcinoma group, however this decrease was not statistically significant. This indicates that tumor progression was taking place as noticed with the change in body weight, whereas DOX due to its anticancer effects reduced the tumor development and resulted in maintenance of the animal body weight (Fig. 10.3).
Fig. 10.3. Effect of mammary cancer and DOX on % IBW. Data represents mean ± SEM of % increase in body weight, n=12.

10.4.4. Haematological Profiling

No significant difference was noticed for haemoglobin, RBC, WBC counts although there was a relative decrease in the hemoglobin levels and RBC, WBC cell counts in DOX treated group with respect to tumor control group (Table 10.2).

Table 10.2. Effect of mammary cancer and DOX treatment on haematological profile.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RBC (×10^6 cells /µl)</th>
<th>Hb % (g/dl)</th>
<th>WBC (×10^3 cells /µl)</th>
<th>Granulocytes (×10^3 cells /µl)</th>
<th>Lymphocytes (×10^3 cells /µl)</th>
<th>Monocytes (×10^3 cells /µl)</th>
<th>Platelets (×10^3 cells/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary tumor control</td>
<td>7.09 ± 0.15</td>
<td>10.86 ± 0.17</td>
<td>7.08 ± 0.68</td>
<td>1.21 ± 0.10</td>
<td>7.04 ± 0.55</td>
<td>1.11 ± 0.10</td>
<td>538.11 ± 23.55</td>
</tr>
<tr>
<td>DOX control</td>
<td>6.93 ± 0.23</td>
<td>10.81 ± 0.16</td>
<td>6.98 ± 0.48</td>
<td>1.03 ± 0.07</td>
<td>6.87 ± 0.46</td>
<td>1.05 ± 0.05</td>
<td>521.11 ± 22.50</td>
</tr>
</tbody>
</table>

Notes: Data represents mean ± SEM of various haematological parameters, n=12

10.4.5. Open field test

Distance travelled and the average speed of animals in tumor control and DOX + tumor groups were not significantly different which indicates that neither mammary carcinoma nor DOX treatment has negative influence on locomotor activity. This supports the fact that, cognitive assessment for episodic and spatial memory components is ideal without the confounding influences of inhibitory effects of either cancer challenge or chemotherapy with DOX (Fig.10.4).
Fig. 10.4. Effect of mammary tumor and DOX on locomotor activity. Data represents mean ± SEM of (a) Mean distance travelled and (b) Mean velocity using an open field task (n=12).

10.4.6. Tumor volume

Group treated with DOX for 10 cycles has shown significant reduction in tumor volume as compared to mammary tumor control group. This shows that DOX is effective in controlling the tumor progression and has potential anticancer activity to treat mammary carcinoma (Fig.10.5).

Fig. 10.5. Effect of DOX on mammary tumor volume. Data represents mean ± SEM of tumor volume which was measured using Vernier calipers, ***p<0.001 (n=12).
10.5. Discussion

In previous chapter, we observed the fact that, mammary carcinoma did not affect cognitive function as compared with age matched healthy control group. Hence it could be the deteriorating effect of chemotherapeutic agents rather than mammary cancer itself, underlying chemobrain associated cognitive decline and the accompanying behavioural dysfunction.

In this present chapter, we have assessed the influence of DOX on cognitive functioning in mammary carcinoma bearing animals which may reflect the most relevant animal model for chemobrain condition which is observed clinically in cancer survivors. As the condition is highly reported in breast cancer survivors and in survivor population who underwent chemotherapy, we assessed DOX for its chemobrain-inducing potential in mammary carcinoma rats which may correlate with human breast cancer.

ORTH and MWM tasks were used to assess the episodic and spatial memory components respectively as we evaluated in the previous chapters. The findings of cognitive functional assessment in tumor control animals was similar to as that noticed in previous chapter (Chapter 9), i.e. mammary carcinoma bearing animals did not show any deficits of either episodic or spatial memory functioning. However, when these mammary cancer bearing rats underwent 10 cycles of DOX chemotherapy, a significant decline in episodic as well as spatial working memory was noticed in object recognition task and Morris water maze tasks respectively.

Mammary cancer rats treated with DOX have spent almost equal time with familiar and novel objects whereas tumor control group has spent significantly more time exploring novel object as compared to familiar object. Further significant decrease in RI and DI was noticed for the group treated with DOX chemotherapy when compared to vehicle treated tumor control group. This indicates that, DOX has negative impact on episodic memory function in cancer condition.

In case of MWM task also, tumor control group was able to form spatial acquisition or retrieval memories, however DOX chemotherapy has resulted in significant deterioration of spatial acquisition or retention form of memories.

It was found that, locomotor activity was not affected by either mammary carcinoma or DOX chemotherapy which further supports the validity of evaluating the cognitive parameters using ORT and MWM tasks. Tumor volume was significantly reduced with DOX chemotherapy over 50 days and no mortality was noticed during the study period which supports the therapeutic efficacy of DOX for the treatment of breast cancer.
Although there was a relative decrease in hematological parameters, viz., Hb% and cell counts, these differences were not significant. This indicates that, modest myelosuppressive effect was noted following 10 cycles of DOX therapy.

Findings from this chapter infer that, DOX has a high negative impact on complete behavioural function especially for cognitive machinery in the existing cancer stage, at least when the mammary cancer stage is an existing condition. This is because, in the previous chapter we noticed that DOX treatment has affected only one tested memory component (episodic memory in ORT), but did not affect spatial memory when the animals are completely healthy without any existence of cancer.

Therefore, it may be confirmed that, chemotherapy can further worsen the cognitive processing and negatively affects new memory formation and retrieval to the maximum extent that it can do when there is an existing cancer state.

Furthermore, it was reported that, cancer may activate body's immune system with the resulting cytokine dysregulation and the inflammatory response can influence cognitive function negatively.

Hence we confirm that, preclinically it will sound good when we make use of animal models of cancer forms which are more clinically relevant and thereby to test chemotherapeutic drugs for their potential chemobrain associated side effects by effectively controlling many of the factors which are generally cannot be controlled clinically to study the chemobrain phenomenon.

**10.6. Conclusion**

DOX on long-term treatment has negative effects on cognitive function and in addition, this deteriorating effect was further worsened in the presence of cancer, i.e., in mammary carcinoma bearing animals. However, DOX has therapeutic utility to reduce the progression of NMU-induced mammary carcinoma.

Hence it was concluded that, for exploring the chemobrain related side effects of cytotoxic agents used to treat various cancers, animal models which make use of cancer animals are more sensitive, ideal with a better clinical relevance and reproducibility.