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Evaluation of Naringin and Rutin to Reverse
Doxorubicin-Induced Chemobrain in Mammary
Cancer Rats

11.1. Introduction
Most of the clinical reports investigating chemobrain complications were observed in breast cancer (BC) survivors due to their remarkably enhanced survival rate. Hence we have focused on developing the most relevant animal model involving mammary carcinoma bearing animals in earlier chapters. Major goal of the present work is to identify the negative effects of chemotherapy on cognitive behaviour and thereby to explore probable interventions in the rat model of mammary carcinoma developed followed by correlating the findings with the clinically observed chemofog complications in BC survivors.
In the earlier chapters, we confirmed that rat model of mammary carcinoma, rather than models using healthy animals could be a clinically relevant and ideal animal model for exploring the chemofog complications observed with DOX chemotherapy.
We initially observed that, test flavonoids NAR and RUT have shown promising procognitive properties in preliminary animal models of cognitive deterioration induced by time delay or by scopolamine published recently (Ramalingayya et al., 2016). Further, they protected against DOX-induced cognitive decline in rats treated with DOX.
It is also important that, NAR and RUT suppress the toxicity of DOX without affecting the anticancer potential of DOX. Hence further study was designed to evaluate the protective effect of NAR and RUT against DOX in NMU-induced mammary carcinoma rats.

11.2. Objectives
Primary objective
To evaluate NAR and RUT to reverse the DOX-induced cognitive deficits in rat model of mammary carcinoma.
Secondary objective
To assess whether the anticancer potential of DOX chemotherapy against mammary cancer is influenced by flavonoids co-treatment.

11.3. Materials and Methods
11.3.1. Animals
Female mammary carcinoma bearing rats weighing, 200-250 g were used in this present study. Female Sprague-Dawley rats (when the age was 30-35 days) were procured from
central animal research facility of Manipal University, Manipal to induce mammary carcinoma by using carcinogen, i.e. NMU. All the experimental procedures were approved by IAEC.

11.3.2. Experimental design
Initially, mammary cancer was induced by N-nitroso, N-methyl, urea (NMU) as per the procedures described in previous chapter 8, page 79. Following the induction of mammary carcinoma, mammary cancer bearing animals were randomized and grouped into four groups consisting 9 rats in each group, based on the tumor volume and number of tumors.

Group 1 was mammary tumor control group which received intraperitoneal saline injections once in every 5 days and daily oral treatment with vehicle (0.25% w/v CMC). Group 2 was DOX control group and received intraperitoneal DOX (10 cycles at 2.5 mg/kg, i.p.) once in every 5 days along with daily vehicle (0.25% w/v CMC) treatment orally. Group 3 and 4 were test flavonoid treatment groups which received DOX (10 cycles at 2.5 mg/kg, i.p.) along with either NAR or RUT formulation in 0.25% w/v CMC at a dose of 50 mg/kg, p.o. daily respectively. Dose volume employed was 2 ml/kg throughout all the treatment schedules.

11.3.3. Formulations and treatments
NAR and RUT were formulated as suspensions in 0.25% w/v sodium CMC in double distilled water. DOX was made as a solution in normal saline from a stock of 2 mg/ml.

11.3.4. Experimental parameters monitored
Exploration time, DI and RI in ORT; Escape latency, path length, swim speed, Q4 time and Q4 latency in MWM; % change in body weight, acetylcholinesterase, hematological profiling, and oxidative stress markers as mentioned earlier. The procedures for these are described already in previous chapters.

11.3.5. Organ index
Following necropsy, all the major organs were collected and weight of the organ per 100 g of rat body weight was calculated as organ index and compared to assess organ level toxicity.

11.3.6. Mammary tumor profiling
Mammary tumor volume was assessed at the start of the study and then following behavioural analysis before termination of study using digital Vernier calipers.

11.3.7. Histopathological analysis
Animals were perfused with 10% v/v neutral buffered formalin (NBF) and tumor samples as well as major organ systems cerebral cortex, heart, liver were fixed in 10% v/v NBF and processed in gradient alcohol and xylene followed by paraffin fixation overnight. Sections were cut using microtome and processed for routine H & E staining. Sections were then
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mounted in DPX and observed under light microscope and images were captured under 40X magnification.

11.3.8. Statistical analysis

Statistical analysis was carried out for all the parameters monitored as mentioned in chapter 7, page 61.

11.4. Results

11.4.1. Protective effect of NAR and RUT on DI and RI in ORT against DOX-induced cognitive dysfunction in mammary carcinoma rats

Vehicle treated mammary cancer rats were able to remember familiar object and spent significantly more time with novel object as compared to familiar one, hence showed discrimination following an ITI of 2 h. However, DOX and vehicle treated animals have spent almost equal time with either familiar or novel object which indicates that, these animals were not able to remember the familiar object without any discrimination between the objects.

NAR co-administration along with DOX did not result in protection from chemobrain induced by DOX as these animals did not show any discrimination between the objects during choice trial. However, treatment with DOX along with RUT has resulted in significant protection against DOX-induced chemobrain associated cognitive deterioration for episodic memory as the animals have spent significantly more time exploring novel object during choice trial.

Further, significant increase in discriminative and recognition indices were observed for the group treated with DOX and RUT as compared to DOX control group. This indicates that, co treatment with RUT offered significant protection against DOX-induced chemobrain in view of episodic memory and cognitive processing (Fig.11.1).
**Fig. 11.1.** Effect of DOX and test flavonoids on exploration time of either familiar or novel objects, recognition and discriminative indices during choice trial of ORT. Data represents mean ± SEM of (a) Object exploration time, **p<0.01, ***p<0.001** vs. familiar object, (b) Recognition and (c) Discriminative indices during ORT testing, **p<0.01** vs. DOX control (n=9).

### 11.4.2. Protective effect of NAR and RUT on spatial acquisition or retention memory against DOX-induced cognitive dysfunction in mammary carcinoma rats

Mammary tumor animals treated with vehicle, *p.o.* have learned information regarding the location/orientation of platform using the spatial cues over a period of four days of acquisition trials (from day 1-4). However tumor bearing rats treated with DOX for 10 cycles has resulted in deficits of spatial acquisition learning as there was a significant increase in escape latency for this group (starting from day 2) as compared to the vehicle control group (Fig.11.2).

Testing track plots were shown in Fig.11.3 which gives an idea about the path taken by animals on different days of acquisition trials from day 1 to day 4.
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**Fig. 11.2.** Effect of DOX and test flavonoids on spatial acquisition learning and memory in MWM test. Data represents mean ± SEM of (a) Target latency, (b) Path length and (c) Swim speed during acquisition trials of MWM testing (n=9), *p<0.05, **p<0.01 and ***p<0.001 vs. DOX control.
Fig. 11.3. Test track plots for the effect of DOX and test flavonoids on spatial acquisition learning and memory in MWM test. Blue dot indicates track start point and red dot indicates track stop point.

Further, we observed a significant reduction in Q4 time for DOX control group as compared to vehicle control which indicates that, this group has spatial retention memory deficits as well (Fig.11.4). Co-treatment with NAR at 50 mg/kg, p.o. did not protect from DOX-induced spatial memory deficits as no significant improvement was noted for any of the acquisition or retention memory parameters. However, RUT co-treatment with DOX resulted in significant improvement of spatial acquisition and retention learning measures in MWM test as compared to DOX control. This infers that, chronic treatment with RUT (50 mg.kg, p.o.) protected from DOX-induced spatial learning deficits associated with clinical chemobrain like condition.
Furthermore, the swim speed was found to be not significantly different among treatment groups which supports that the animal model used for spatial memory was not influenced by the presence of tumor or change in locomotor activity.

Fig. 11.4. Effect of DOX and test flavonoids on spatial retention memory in MWM test. Data represents mean ± SEM of (a) Time spent in target quadrant/ Q4 Time, **p<0.01 vs. DOX control (b) Q4 Latency during retention or probe trials of MWM testing (n=9).

11.4.3. Change in body weight

It was found that, tumor vehicle control animals and the group treated with DOX + NAR started losing body weight from treatment day 49 onwards, whereas steady growth of animal weight was noted in case of other groups of animals, i.e. DOX control or DOX + RUT group. This shows that, tumor progression was evident in tumor control group and either DOX (chemotherapy) or flavonoid, RUT may be protecting from the loss of body weight as a result of nutrition deficit due to tumor development (Fig.11.5).
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Fig. 11.5. Effect of DOX and test flavonoids on % IBW. Data represents mean ± SEM of % change in body weight throughout the treatment period, **p<0.01, ***p<0.001 vs. vehicle control (n=9).

11.4.4. Haematological profiling

Tumor control and DOX control animals had shown myelosuppressive effects in view of RBC, WBC counts along with a reduction in Hb%. However co-treatment with RUT significantly protected from myelosuppressive effect induced by DOX and mammary tumor (Table 11.1).

Table 11.1. Effect of flavonoids on mammary carcinoma and DOX-induced myelosuppression.

<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>Vehicle tumor control</td>
<td>6.58 ± 0.45</td>
<td>10.46 ± 1.12***</td>
<td>8.68 ± 0.81***</td>
<td>1.18 ± 0.10***</td>
<td>7.22 ± 0.68***</td>
<td>1.21 ± 0.11***</td>
<td>587 ± 43.55***</td>
</tr>
<tr>
<td>DOX control</td>
<td>5.12 ± 0.36</td>
<td>8.41 ± 0.96</td>
<td>4.01 ± 0.54</td>
<td>0.82 ± 0.06</td>
<td>5.14 ± 0.48</td>
<td>0.95 ± 0.15</td>
<td>221 ± 22.50</td>
</tr>
<tr>
<td>DOX + NAR</td>
<td>8.15 ± 0.64**</td>
<td>11.82 ± 1.01</td>
<td>6.76 ± 0.66</td>
<td>1.09 ± 0.2</td>
<td>7.12 ± 0.67</td>
<td>1.10 ± 0.09</td>
<td>610 ± 51.11***</td>
</tr>
<tr>
<td>DOX + RUT</td>
<td>9.43 ± 0.61***</td>
<td>14.62 ± 1.19**</td>
<td>7.45 ± 0.69**</td>
<td>1.32 ± 0.11</td>
<td>7.33 ± 0.66</td>
<td>1.20 ± 0.11</td>
<td>677 ± 47.19***</td>
</tr>
</tbody>
</table>

Notes: Data represents mean ± SEM of haematological parameters at the end of the study period, **p<0.01, ***p<0.001 vs. DOX control (n=9)
11.4.5. Acetylcholinesterase (AChE) activity

AChE activity was found to be almost similar across the four treatment groups without any significant differences either in hippocampus or frontal cortex (Fig. 11.6).

![Fig. 11.6. Effect of DOX and test flavonoids on acetylcholinesterase (AChE). Data represents mean ± SEM of enzyme activity in (a) Hippocampus and (b) Frontal cortex in terms of micromoles of acetyl thiocholine iodide hydrolysed per min per mg of protein (n=9).](image)

11.4.6. Oxidative stress

Oxidative stress was significantly elevated in frontal cortex and hippocampus regions in DOX control group as compared to vehicle tumor control. However, treatment with RUT along with DOX alleviated the elevated oxidative stress and significantly protected from oxidative damage. Further, NAR could not prevent the DOX-induced oxidative stress and the levels of catalase, SOD, GSH and total thiols were not significantly different from DOX control in frontal cortex and hippocampus (Fig. 11.7 & Fig. 11.8).
Fig. 11.7. Effect of DOX and test flavonoids on oxidative stress markers in hippocampus. Data represents mean ± SEM of (a) Catalase, (b) SOD, (c) GSH and (d) Total thiols in hippocampus of brain, *p<0.05, **p<0.01, ***p<0.001 vs. DOX control (n=9).
11.4.7. TNF-alpha levels

TNF-alpha levels were significantly elevated in frontal cortex and hippocampal regions for DOX control group as compared to vehicle tumor control group. However this elevated neuro-inflammatory marker as a result of DOX chemotherapy was significantly averted by RUT co-treatment, but not by NAR in both frontal cortex and hippocampus regions (Fig.11.9).
Fig. 11.9. Effect of DOX and test flavonoids on TNF-alpha in hippocampus and frontal cortex. Data represents mean ± SEM of TNF-alpha levels in (a) Hippocampus and (b) Frontal cortex, *p<0.05, **p<0.01 vs. tumor control and ###p<0.001 vs. DOX control (n=9).

11.4.8. Organ index

Organ index was found to be similar for all the major organs evaluated and no significant changes were observed across the treatment groups. This shows that, organ level changes were not significantly evident with the tumor challenge or the various treatments employed (Fig.11.10 & Fig. 11.11).
Fig. 11.10. Effect of DOX and test flavonoids on organ index part-I. (a) Liver, (b) Heart, (c) Thymus, (d) Spleen, (e) Left adrenal and (f) Right adrenal. Data represents mean ± SEM of organ indices (n=9).
Fig. 11.11. Effect of DOX and test flavonoids on organ index part-II. (a) Left kidney, b) Right kidney, c) Brain, d) Left ovary and e) Right ovary. Data represents mean ± SEM of major organ indices (n=9).

11.4.9. Mammary tumor volume

Tumor volume was found to be significantly \( p<0.001 \) elevated for vehicle tumor control group as compared to DOX control at the end of the study period. Further, rats treated with DOX in combination with either NAR or RUT showed no significant increase in tumor volume as compared with DOX control group which suggest that, antitumor efficacy of DOX was not hindered or enhanced by treatment with flavonoids, i.e. NAR and RUT (Fig.11.12). Moreover, the number of mammary tumors was not different among the groups treated with
DOX alone or in combination with test flavonoids. Furthermore, none of the groups showed mortality till the end of the study period and the animals were found to be actively moving throughout the study period.

![Tumor volume graph](image)

Fig. 11.12. Effect of DOX and test flavonoids on mammary tumor volume. Data represents mean ± SEM of tumor volume across various treatment groups, *p<0.001 vs. tumor vehicle control (n=9).

11.4.10. Histopathological analysis

11.4.10.1. Mammary tumors

Mammary tumor vehicle control animals showed severe carcinogenesis of mammary gland with ductal and lobular adenocarcinoma along with highly proliferating epithelial cells and infiltration of lymphocytes, while DOX treated tumor samples showed diminished tumor progression with moderate ductal carcinoma along with reduced infiltration of leukocytes and lessened proliferation of epithelial cells. Treatment with NAR and RUT did not aggravate the tumor progression when given along with DOX as there was no difference in histological characters when compared to DOX control samples (Fig.11.13.1)

11.4.10.2. Cerebral cortex

Cerebral cortex of mammary tumor control animals found to have healthy architecture with no marked gliosis, whereas DOX control rats showed severe neurodegeneration of cerebral cortex and gliosis which indicates neuro-inflammation. Treatment with either NAR or RUT along with DOX protected from DOX-induced cerebral neurodegeneration (Fig.11.13.2).

11.4.10.3. Heart

Cardiac tissue of mammary tumor control animals showed normal histological features of myocardial tissue, while DOX control rats exhibited myocardial degeneration along with marked necrotic areas which indicates cardiotoxicity. Treatment with NAR and RUT along
with DOX respectively protected from DOX-induced myocardial degenerative changes showing their potential cardioprotective activity (Fig.11.3.3).

11.4.10.4. Liver

Normal histological architecture of liver hepatocyte parenchyma observed with mammary tumor control animals. DOX control rats showed hepatocyte degeneration along with portal vein dilation which indicates hepatotoxicity whereas treatment with NAR and RUT along with DOX respectively protected from DOX-induced hepatocyte degeneration showing their possible hepatoprotective activity (Fig.11.3.4).

Fig. 11.3. Histopathological analysis of mammary tumors (11.3.1.), cerebral cortex (11.3.2.), heart (11.3.3.) and liver parenchyma (11.3.4.). A- Mammary tumor control, B- Mammary tumor rats treated with DOX, C- Mammary tumor rats treated with DOX and NAR, D- Mammary tumor rats treated with DOX and RUT. Tumor vehicle control animals showed evidence of ductal and lobular adenocarcinomas of mammary glands with rapidly proliferating epithelial cells. Presence of fibro adenomas was also evident. However, DOX treatment has resulted in lessened proliferating epithelial cells with diminished progression of carcinomas. Treatment with flavonoids, i.e. NAR and RUT did not result in alteration of histological features observed with DOX. DOX-induced pathological abnormalities to major organ systems, cerebral cortex, heart and liver were alleviated by co-treatment with RUT, but moderate protection was noticed with NAR.
11.5. Discussion

In this present chapter, we evaluated the possible role of natural flavonoids, NAR and RUT as therapeutic interventions to protect from DOX-induced chemobrain (based on the preliminary promising nootropic potential in animal models of dementia and chemobrain in healthy animal model developed in our laboratory) in a clinically relevant animal model of mammary carcinoma. Further, to know whether these flavonoids have influencing effects on antitumor activity of DOX, we assessed the mammary tumor profiling across all the treatment groups.

In the previous chapter, we found that, DOX was worsening the cognitive function further in presence of mammary tumor as compared to healthy animals. Hence, we confirmed that the use of cancer animal model would be ideal for assessment of chemobrain complications observed clinically in BC survivors.

We found that, DOX significantly produced episodic and spatial memory deficits as noted with ORT and MWM tests respectively. Further, no deficits were observed with tumor control group which indicates that, DOX was responsible for the learning and memory deficits noticed in mammary tumor bearing animals. However, treatment with RUT but not NAR, has significantly alleviated episodic and spatial memory deficits and offered protection against DOX-induced cognitive deterioration. Further, it was evident that anticancer activity of DOX was not hindered by co-treatment with test flavonoids which further supports the use of natural flavonoids as adjuvant therapy along with chemotherapy to protect from chemobrain associated cognitive complications so as to improve the QOL in cancer survivors, especially the BC survivors.

In addition, the body weight changes were not significantly changed among the groups treated with DOX and DOX + RUT which indicates that, RUT was able to protect from DOX-induced chemobrain without interfering with antitumor efficacy of DOX.

Further, the levels of AChE enzyme activity were not significantly different among the treatment groups which indicates that the neurobiology of chemobrain condition is unlike Alzheimer's disease type dementia.

Oxidative stress has been significantly elevated in frontal cortex and hippocampus for the DOX control group. This has been significantly averted by daily co-treatment with RUT but not NAR which infers the potential antioxidant mechanism of flavonoid, RUT against DOX-induced oxidative stress and the associated cognitive deterioration.
We found that, TNF-alpha was significantly elevated in DOX control group as compared to tumor control. However, this was significantly alleviated by RUT which may be attributed to its potential anti-inflammatory activity (Koda et al., 2009). Further, treatment with flavonoid RUT significantly protected from myelosuppressive effects of mammary tumor and DOX chemotherapy in view of RBC and WBC counts along with Hb%. This could be one of the mechanism underlying the protective effect of RUT against DOX-induced chemobrain as reported by the earlier study (Fan et al., 2009).

Also it was noted that, organ index was not significantly varying throughout the experimental groups which reflects the absence of gross organ changes with either tumor or treatments. However, histological analysis of mammary tumor samples revealed that there was formation of ductal, lobular adenocarcinomas and fibro adenomas with rapid proliferation of epithelial cells. Treatment with either DOX alone or in combination with NAR or RUT has shown similar histological characteristics which indicates that, flavonoid treatment did not interfere with the DOX chemotherapy against mammary tumor. Further, DOX-induced pathological abnormalities to the major organ systems, i.e. cerebrum, heart and liver were protected by co treatment with RUT, but NAR treatment had lesser influence on it. This further proved that the potential of RUT in alleviating the structural changes of major organs systems as well which can have a great positive impact on cancer survivors health related QOL for betterment of ADL.

11.6. Conclusion

We conclude that, RUT but not NAR has protective potential against DOX-induced chemobrain accompanying cognitive deterioration for episodic and spatial memory processes in the developed and most relevant animal model of chemobrain, i.e. rat model of mammary carcinoma. This could be attributed to RUT's potential to alleviate cellular oxidative stress, neuro-inflammation, intrinsic nootropic potential and organ protective effects. Further, both the flavonoids, NAR and RUT did not interfere with the antitumor efficacy of DOX in view of tumor volume and progression.
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Bibliography

