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Rat Model of Mammary Carcinoma

8.1. Introduction
Most of the preclinical animal models working on potential breast cancer therapeutics make use of rodent models of mammary carcinoma. Mammary cancer is generally induced in rodents by chemical carcinogens which may possibly replicate the breast cancer (external factors-induced) in humans. Preclinically, most common chemical carcinogens used to induce mammary cancer are di-methyl benzanthracene (DMBA), N-nitroso N-methyl urea (NMU) etc.

Some of the cancer studies in BALB/c mice make use of implanting biological cell lines (for e.g. 4T1 etc.) in immunocompromized nude animals which can induce vigorous type of mammary cancer within a very short period of latency in rodents (Kaur et al., 2012). However, they have high mortality rates (Tai et al., 2014) because of high tumor metastatic nature which may not be suitable for studying and modeling chemobrain accompanying neurocognitive complications at preclinical level. Further, animals need to be exposed to the external environment which is not advisable. Hence we have used chemical-induced mammary carcinoma in this study.

We have selected NMU as chemical carcinogen to induce the mammary cancer as literature supports the fact that, NMU treatment results in the most relevant forms of human breast cancer (involving the resemblance for ductal adenocarcinomas) as compared to the other chemicals like, DMBA etc.

Furthermore, NMU has the advantage of cost effectiveness, single dose administration, shorter tumor latency with high incidence rates (around 75%) along with very low mortality rates. It is of utmost importance to have shorter tumor latency as the age of animals may become a confounding influence especially in case of assessing cognitive function in rodents. Moreover, aged animals are more prone to death due to mammary carcinoma. The shorter tumor latency observed with NMU administration has the advantage of excluding the influencing factors related to age for assessing cancer and chemotherapy-induced behavioural dysfunction for cognitive deterioration.
8.2. Objectives

Primary objective- To induce mammary cancer in female rats with NMU

Secondary objective- To validate the dose, frequency and route of administration by establishing the mammary cancer induction with shorter tumor latency and high incidence rates with reproducibility.

8.3. Materials and Methods

8.3.1. Animals

Female Wistar and SD rats separated after weaning period, aged 30-35 days weighing 50-80 g were procured from CARF of Manipal University, Manipal. All the experimental procedures were approved by IAEC (Institutional Animal Ethics Committee) with approval number, IAEC/KMC/17/2013.

8.3.2. Chemicals

N-nitroso, N-methyl urea (NMU) was procured from Sigma Aldrich chemicals, USA and stored at -20°C until use. 3% v/v acetic acid (Merck chemicals, Mumbai) was used to maintain the acidic pH of about 4.0. Normal saline which was used to make the NMU solution was also obtained. All other chemicals used in this study were of reagent grade.

8.3.3. Experimental design

Total of five groups (n=6 each) of female nulliparous rats were used to assess the mammary cancer induction. Group-1 was a healthy control group treated with normal saline, whereas the Group 2 & 3 were treated with single injection of NMU at 50 mg/kg by i.p. and i.v. routes of administration respectively.

Separately two groups (Group 4 & 5) of animals were assigned for testing multiple (dosing for twice with one week dosing interval) injections of carcinogen at 50 mg/kg by i.p. and i.v. routes of administration respectively for any enhancement of tumor latency or incidence rate without producing the mortality.

Tumor induction was evaluated in both Wistar and SD strains of rats as the literature reported most of the preclinical mammary carcinoma studies in Wistar and SD rats.

8.3.4. Formulations and treatments

NMU is highly hygroscopic and thermo labile carcinogen in nature. NMU was formulated as a clear uniform slight yellow colored solution in acidified (pH-4.0) vehicle, i.e. normal saline at a dose of 50 mg/kg. The purpose of acidified saline is to activate the carcinogen in vivo once administered into biological system.

Normal saline was acidified with 3% v/v acetic acid and then, pH was adjusted to 4.0 with diluted acetic acid. NMU is soluble in this acidified vehicle. NMU was prepared just prior to
administration and the entire process of carcinogen administration in vivo was completed within 30 min of its preparation in acidified saline.

When the female rats were about 30-35 days old in the weight range of 50 - 80 g, carcinogen administration was taken place. Group 1 received normal saline, whereas Group 2 & 3 were administered with single NMU injection at 50 mg/kg, by i.p. and i.v. routes respectively. Group 4 & 5 received two doses of NMU at 50 mg/kg, by i.p. and i.v. routes respectively with a dosing interval of one week.

8.3.5. Assessment of mammary carcinoma development

Earlier reports showed that, NMU-induced mammary cancer can take 8-12 weeks depending on the animals' age at which NMU is administered, route and frequency of carcinogen administration (McCormick et al., 1981; Pyter et al., 2010). From week 4 onwards from the date of carcinogen administration, animals were individually placed in cages and mammary glands were palpated daily for any growth of tumor like development. Only those tumors in the vicinity of mammary gland which were of diameter more than or equal to 4 mm were considered as mammary tumors. Tumor latency, incidence rate, number of tumors developed per animal was noted and compared among various groups. Mortality, if any was also reported.

When the animals started developing the tumors, the volume of tumors was assessed i.e., major and minor diameters were measured for tumor volumetric calculations.

8.3.6. Histopathology of mammary carcinoma development

The developed mammary gland tumors were isolated in intact structures following the euthanasia by making use of excess ketamine. Tumor samples were processed with usual graded ethanol and xylene treatments and fixed overnight in liquid paraffin. The coronal sections of mammary tumors were taken for histopathological analysis by eosin & haematoxylin (H&E) staining method as described in chapter 7, page 60 & 61.

8.4. Results

8.4.1. Assessment of mammary carcinoma development

None of the tested groups of Wistar rats (whether single or multiple injections of NMU by i.p./i.v. routes of administration) have developed mammary tumors, even up to 1 year of observation period following which they were euthanized by excess of ketamine.

However, SD strain rats have developed mammary carcinoma 2-3 months following NMU administration. All the SD rats which have developed tumors have started developing the carcinomas within the time line of 30 days, i.e. 65 days to 95 days from the date of carcinogen administration.
8.4.1.1. Tumor latency

All the rats of Group 1 which received saline treatment did not show any signs of growth for tumor development in the region of mammary glands throughout the study period which infers that the condition of the animals was healthy.

Group 2 animals which received single injection of NMU at 50 mg/kg by intraperitoneal route have developed mammary carcinoma in the region of mammary gland. Tumor latency for this group was found to be in the range of 69-84 days with an average of 75 days.

Group 3 animals which received single injection of NMU at 50 mg/kg by intravenous route have developed mammary carcinoma in the region of mammary gland. Tumor latency for this group was found to be in the range of 86-93 days with an average of 90 days.

With multiple doses of carcinogen administration by i.p. or i.v. routes in group 4 & 5 respectively, no further enhancement of tumor progression, i.e. reduction in tumor latencies was not observed. The average tumor latency was found to be 74 & 88 days respectively for these groups. Furthermore, reduction in body weight was found to be more with multiple injections along with some co-morbidity signs.

8.4.1.2. Tumor incidence rate

5 rats out of 6 animals in group 2 were found to develop mammary carcinoma. The incidence rate for group 2 animals was observed as 83.3%. In group 3, about 4 out of 6 animals have developed tumors which indicates an incidence rate of 66.7%. Similarly for group 4 & 5, incidence rates were noticed at 66.7 & 50% respectively.

8.4.1.3. Tumor frequency

Most of the animals have developed single tumors in the vicinity of mammary glands. For group 2, the total number of tumors developed were 5 with a maximum of 1 tumor per animal whereas the tumor frequency was 4 for group 3 with a maximum of 1 tumor per animal. The tumor frequency was found to be 4 and 3 for the groups 4 & 5 respectively with a maximum of 1 tumor per animal.

8.4.1.4. Tumor volume

Tumor volume was measured on a cut off day, i.e. day 100 from tumor induction, the time by which animals have developed the tumor. The mean tumor volume was found to be 1.80 & 1.68 cm³ for group 2 and 3 respectively. Also for group 4 & 5 with multiple carcinogen administrations, the average tumor volume was noted at 1.45 & 1.51 cm³ respectively.

Tumor profiling results were mentioned in Table 8.1.
Table 8.1. NMU-induced mammary tumor assessment parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor latency (days)</th>
<th>Tumor incidence rate (%)</th>
<th>Tumor frequency per animal</th>
<th>Tumor volume (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline injection, 2 ml/kg, i.p.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Single NMU injection, 50 mg/kg, i.p.</td>
<td>75 ± 2.92</td>
<td>83.33</td>
<td>1</td>
<td>1.80 ± 0.24</td>
</tr>
<tr>
<td>Single NMU injection, 50 mg/kg, i.v.</td>
<td>90 ± 1.47</td>
<td>66.67</td>
<td>1</td>
<td>1.68 ± 0.34</td>
</tr>
<tr>
<td>Multiple NMU injection, 50 mg/kg, i.p.</td>
<td>74 ± 2.71</td>
<td>66.67</td>
<td>1</td>
<td>1.45 ± 0.12</td>
</tr>
<tr>
<td>Multiple NMU injection, 50 mg/kg, i.v.</td>
<td>88 ± 1.15</td>
<td>50.00</td>
<td>1</td>
<td>1.51 ± 0.15</td>
</tr>
</tbody>
</table>

Notes: Data represents tumor assessment parameters among the five tested groups

8.4.2. Histopathological analysis

With H & E staining of tumor samples, we observed that the growth in mammary gland region was due to induction of mammary tumor. It was also confirmed that, the development of mammary tumor involved ductal carcinoma which generally believed to be human relevant ductal adenocarcinoma of breast (Fig. 8.1).

Fig. 8.1. Illustration represents N-nitroso, N-methyl urea-induced rat model of mammary carcinoma; (a) Mammary tumor in intact animal, (b) Histopathological identification of mammary tumor features, i.e. ductal adenocarcinoma and increased proliferation of epithelial cells with dilated ducts represented by arrows (H& E staining, 40X).
8.5. Discussion

In this present chapter, procedure for NMU-induced mammary cancer has been validated as we have performed the tumor induction for the first time in our laboratory. The strain of animals as well as dose, frequency and route of administration was validated with reliability and reproducibility. We found that, Albino Wistar rats did not develop mammary tumors despite of multiple carcinogen (NMU) administration which indicates that, Wistar strain animals are resistant to the NMU-induced mammary cancer development. However SD rats have developed mammary carcinoma following either single or multiple carcinogen injections. Further, SD rats showed growth of mammary tumors with either intraperitoneal or intravenous route of administration. Relatively, single NMU injection by i.p. route has resulted in shorter tumor latency (75 days), improved incidence rate (83%) as compared to either i.v. carcinogen administration or multiple carcinogen administrations. Furthermore, multiple carcinogen administration has resulted in signs of morbidity which will become confounding influence for behavioural assessment.

No mortality was noted across all the treatment groups of SD or Wistar rats which supports the use of this chemical, i.e. NMU for slow and steady progression of tumor development. Age of the female rats at which the carcinogen was administered plays a very important role in tumorigenesis. In our present study, 30-35 days old animals were selected for carcinogen administration as suggested by earlier reports (Pyter et al., 2010). Histopathological studies revealed that, the growth observed in the vicinity of mammary gland was because of the formation of mammary carcinoma which also confirmed it as a mammary ductal adenocarcinoma. This further correlates with the histopathology of human breast adenocarcinoma for a better clinical relevance.

Hence in all further studies, this procedure was used for carcinogen, i.e. NMU-induced mammary carcinoma in SD rats for more reliability and reproducibility without any comorbidity or mortality.
Bibliography


