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## Histopathology and Immunohistochemical Expression of N-Methyl-N-Nitrosourea (NMU) Induced Mammary Tumours in Sprague-Dawley Rats

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### ABSTRACT

Mammary tumours rank second as the most common neoplasms in dogs after skin tumours, whereas in women the most common cause of cancer-related deaths is breast cancer. N-Methyl-N-Nitrosourea (NMU) is a highly specific mammary gland carcinogen which directly act and does not require metabolic activation. In the present study, NMU at the dose rate of 50 mg kg<sup>-1</sup> body weight was used intra-peritoneally for the induction of mammary tumour. The first palpable tumour appeared on 70th day post carcinogen injection and subsequently, most of the tumours were developed around 18-20th week. During 28 weeks of experimental period, the tumour incidence was 82.86% (29/35). The tumour frequency was found to be 4.7±0.33 tumours and the average latency period was 107±4.1 days. The average tumour volume was found to be 69±8.8 cm<sup>3</sup>. Equally, 50% of mammary tumours appeared on the right (22/44) and left (22/44) mammary gland chain. Region wise, 81.82% (36/44) of the tumours appeared on abdominal-inguinal mammary glands and 18.18% (8/44) on the cervical thoracic mammary glands. A total of 44 mammary tumours were diagnosed in which 88.64% (39/44) were malignant and 11.36% (5/44) were benign. Among the malignant tumours, 33.33% (13/39) were non-invasive and 66.67% (26/39) were invasive. The average values of mitotic index, Proliferating Cell Nuclear Antigen (PCNA), Vascular Endothelial Growth Factor (VEGF) and Platelet Endothelial Cell Adhesion Molecule (PECAM-1) in NMU induced mammary tumours were found to be 4.5±0.46/hpf, 77±2.6, 16.2±0.86 and 15±0.69, respectively. The present study for the first time demonstrated the expression of VEGF and PECAM-1/CD-31 proteins in NMU induced mammary tumours.

**Key words:** N-Methyl-N-Nitrosourea, mammary tumour, histopathology, immunohistochemistry, PCNA, VEGF, PECAM-1, rats

### INTRODUCTION

Cancer remains to be one of the most dreaded ailments for both human and animals, claiming about 7.6 millions of human lives every year (Ferlay *et al.*, 2010), inspite of innumerable

interdisciplinary approaches that have contributed significantly to the progress in cancer diagnosis and treatment. Cancer is the leading cause of mortality in dogs and cats (Bonnett *et al.*, 2005) and second most in humans (1 in every 4 deaths in United States) (Jemal *et al.*, 2008). Mammary tumors are most frequently encountered in dogs, cats and women, although they also occur in other domestic animals with very low frequency. Over one million cases of female breast cancer are diagnosed each year. They constitute about 70% of all neoplasia in bitches (Merlo *et al.*, 2008) and 17% in queens (MacEwen and Withrow, 1996) and 26% in women (Jemal *et al.*, 2008). Mammary tumors are more frequently malignant and they have high tendency of metastasis, recurrence and the median survival time is very short (Martins *et al.*, 2002).

Rodent models have been used as a experimental models for the study of mammary cancer because in this species, the mammary gland tumours are estrogen-dependent, aggressive and locally invasive which is similar to most frequently diagnosed mammary cancer in women in terms of tumour histology and hormone dependence (Gullino *et al.*, 1975; Masso-Welch *et al.*, 2000; Russo and Russo, 2000). When compared with the mouse models, mammary carcinogenesis in rat models are more closely resembles human breast cancer with respect to histopathology, responsiveness to ovarian hormones, protective effects of full-term pregnancy and the absence of a viral etiology (Gusterson and Williams, 1981; Thompson *et al.*, 1995; Perse *et al.*, 2009). The mostly used chemical carcinogen models are N-Methyl-N-Nitrosourea (NMU), 7,12-dimethylbenzanthracene (DMBA), methylchloranthracene (MCA), diethylnitrosoamine (DEN) and azoxymethane (AOM) (Gullino *et al.*, 1975; Macejova and Brtko, 2001; Saminathan *et al.*, 2014). Currently, DMBA and NMU induced rat mammary carcinoma models are widely used to study the human breast cancer. The NMU is a highly specific carcinogen for the mammary gland and in contrast to DMBA it does not require metabolic activation to form DNA adducts and has a very short half-life. The NMU induced mammary tumours are more estrogen dependent, locally aggressive and able to metastasize while the DMBA induced tumours are more prolactin dependent, less aggressive and lack of metastasis. The proportion of benign mammary tumors induced by MNU is lower than with DMBA (Gullino *et al.*, 1975; McCormick *et al.*, 1981; Sukumar *et al.*, 1983; Thompson and Adlakha, 1991).

Chemical carcinogenesis involves a multi-step process that can be divided into the stages of tumour initiation, promotion and progression. The induction of point mutations in critical genes, such as the proto-oncogene c-Ha-ras is the underlying cause of tumour initiation, whereas tumour promotion brought about by tumour promoters such as the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is due to stimulation of expression of genes involved in hyperproliferation, inflammation and reduced expression of genes involved in apoptosis (Sukumar *et al.*, 1983; Miyamoto *et al.*, 1990; Perse *et al.*, 2009). The mechanism of action of direct alkylating agent NMU is conversion of the parent molecule into an active carbonium cation and subsequent alkylation of cellular macromolecules, such as DNA. The molecular mechanism of NMU involves specific G-35 point mutation in codon 12 which results in substitution of normal glycine with an aspartic acid (Sukumar *et al.*, 1983; Miyamoto *et al.*, 1990; Perse *et al.*, 2009). The aim of the present study was easy, cost effective, rapid induction of mammary tumours by using N-methyl-N-nitrosourea in female Sprague Dawley rats and histopathological and immunohistochemical study to assess the behaviour of induced tumour.

## **MATERIALS AND METHODS**

**Experimental animals:** This experiment was approved by the Institute Animal Ethics Committee (IAEC) of Indian Veterinary Research Institute (IVRI), Izatnagar, India and Committee for the

Purpose of Control and Supervision of Experiments on Animals (CPCSEA) before its commencement. The guiding principles in the care and use of laboratory animals together with those described in the declaration of Helsinki and Indian standards were strictly adhered to in the conduct of all the experimental procedures. The out bred, female Sprague-Dawley rats, weighing 25-30 g were obtained from laboratory animal facility, Central Drug Research Institute (CDRI), Lucknow, India at 3 weeks of age. The rats were housed in polypropylene cages in the experimental animal house under environmentally controlled conditions (temperature  $25\pm 2^{\circ}\text{C}$ ; relative humidity 30-70%) with a 12/12 h light/dark cycle. The animals were acclimatized for one week before the commencement of experiments.

**Experimental design:** A total of 55 rats were randomly divided into 2 groups (Group A and B). Group-A (n = 10) received only acidified saline (NMU vehicle), pH 4.0 by intra-peritoneal (i/p) route and served as vehicle control group. Group-B (n = 45) was administered with NMU at the dose rate of  $50\text{ mg kg}^{-1}$  body weight intra-peritoneally (i/p), three doses at 50, 80 and 110 days of age. The whole experiment duration was of 28 weeks.

**Vaginal exfoliative cytology:** All rats were at estrus at the first NMU injection, verified by vaginal exfoliative cytology. This method consists of flushing cells from the vaginal lining by introducing a small amount of fluid (0.2 mL of 0.9% NaCl solution) into the vagina using a pipette and placing one or two drops of the resulting cell suspension onto a labelled glass slide to make a smear. The smears were dried out and fixed in methanol. The slides were stained with Giemsa stain and observed under a light microscope. Rats which were in estrous and pro-estrous NMU carcinogen were administered.

**Experimental induction mammary tumours:** The N-Methyl-N-Nitrosourea (NMU) (Sigma Aldrich, USA) was used as the chemical carcinogen. The NMU injection vials were wrapped with aluminium foil and kept in ice because NMU is sensitive to light and humidity. The NMU was dissolved immediately prior to its use in 4 ml of 0.9% NaCl solution and acidified to pH 4 with acetic acid in such a way that each milliliter was containing 5 mg of NMU and was administered intra-peritoneally (i/p) following all necessary safety and sterile precautions. After dissolving, the NMU was used within 20 min and then the next vial of carcinogen was prepared. The injections were given along the ventral midline of the animal, half way between the third and fourth pair of mammary glands (Gusterson and Williams, 1981; Thompson *et al.*, 1995; Perse *et al.*, 2009).

**Clinical observations:** All animals were observed daily for regular monitoring of clinical status. The weight of the rats was measured weekly throughout the experimental period. Palpation twice a week for detection of any growth in the mammary glands and visible tumour nodules were measured using vernier calipers at regular intervals. Mortality was recorded till the end of experiment. The following parameters were taken into consideration:

- **Tumour induction:** In first day tumour development was recorded
- **Average latency period:** This was determined by the period from carcinogen administration to the appearance of the first tumour in each animal
- **Tumour incidence:** The number of rats carrying at least one tumour and expressed as a percentage

- **Tumour frequency or tumour burden:** The average number of tumours per tumour bearing rat
- **Cumulative number of tumours:** The total number of mammary tumours appeared till the termination of the experiment
- **Tumor volume:** It was calculated using the formula for an ellipsoid tumour:  $V = (4/3) \times \pi \times r_1 \times r_2 \times r_3$  and expressed in  $\text{cm}^3$ . This can also be considered as an estimation of tumour size (Sorensen *et al.*, 2001)
- **Tumour weight:** The weight of the each tumour was measured at the termination of experiment

**Histopathology:** All palpable tumours and suspected areas were collected and fixed in 10% neutral buffered formalin for histopathological and immunohistochemical (IHC) studies. Formalin-fixed tissue samples of 1-2 mm thickness were dehydrated in graded alcohol and cleared in xylene and embedded in paraffin blocks. About 4-5  $\mu$  thick serial sections were taken with rotatory microtome on clean grease free slides and subjected for haematoxylin and eosin staining for histopathological examination (Luna, 1968). Good quality duplicate sections were also taken on 3-minopropyltriethoxysilane (APES) adhesive coated slides for immunohistochemical studies. The H and E stained sections of NMU induced rat mammary tumours were evaluated and tumours were classified according to the Russo and Russo (2000).

**Immunohistochemistry (IHC):** The paraffin sections were deparaffinised and subjected to immunohistochemistry. IHC staining for PCNA, VEGF and PECAM-1 was performed by employing mouse monoclonal anti-PCNA antibody (1:500 dilution) (PC10, sc-56, Santa Cruz Biotechnology, USA), mouse monoclonal anti-VEGF antibody (1:200 dilution) (C-1, sc-7269, Santa Cruz Biotechnology, USA) and goat polyclonal anti-PECAM-1 antibody (1:200 dilution) (M-20, sc-1506, Santa Cruz Biotechnology, USA), respectively. For antigen retrieval, the sections were microwaved in 10 mM tri-sodium citrate buffer (pH 6.0) for 15 min (3 cycles of 5 min each). Endogenous peroxidase activity was quenched by incubating the sections with 3% hydrogen peroxide in methanol for 15 min at Room Temperature (RT) in dark. For blocking of non-specific antigen binding, sections were incubated with 5% normal goat serum (Invitrogen, USA) for 30 min at RT. Then, the sections were incubated with primary antibody for overnight at 4°C. Biotinylated anti-mouse secondary antibody (sc-2017, ImmunoCruz™ mouse ABC Staining System, Santa Cruz Biotechnology, USA) for PCNA and VEGF and anti-goat secondary antibody for PECAM-1 (sc-2023, ImmunoCruz™ goat ABC Staining System, Santa Cruz Biotechnology, USA) was used as per manufacturer's instructions and incubated for 30 min at RT, followed by ExtrAvidin-Peroxidase (sc-2023, ImmunoCruz™ goat ABC Staining System, Santa Cruz Biotechnology, USA), diluted as per manufacturer's instructions and incubated for 30 min at RT. Staining solution was prepared by dissolving 3,3'-diamino benzidine (DAB) and urea-hydrogen peroxide tablets (Sigma-Aldrich, USA) in required volume of deionized distilled water and sections were stained for 3 min at RT. Counterstaining was done with Mayer's hematoxylin. All the steps were interceded by washing thrice (5 min each) in phosphate buffered saline (PBS, pH 7.4). PCNA immunostaining index was evaluated by counting positive and negative nuclei (minimum 1000 neoplastic cells) in 8-10 representative high power fields. Every immunostained nucleus was considered as positive (Pena *et al.*, 1998). VEGF immunostaining index was evaluated by giving a score ranging from 0-2 to each tumour section. Tumours were given score of 0 if there was no staining, score of 1 if there was pale brown staining and score of 2 if there was dark brown staining

(Al-Dissi *et al.*, 2010). PECAM-1/CD-31 immunostaining index was evaluated by counting microvessel density (MVD) using the “hot-spot method”. Slides were first examined at low power (4X) to identify 10 areas with high vessel density followed by re-examination of each area at high power (40X) and counting of all positively stained structures, irrespective of whether a lumen was identified or not. Continuous vessels were counted as 1 vessel and were expressed as count per high-power field in each tumour (Weidner *et al.*, 1991).

**Statistical analysis:** Statistical analysis was performed by using SPSS Advanced Statistics 16.0 software (SPS Inc., Chicago, USA). The body weight data was analyzed by using student's t-test as described by Snedecor and Cochran (1989). All data was expressed as Mean±standard error mean (Mean±SEM).

## RESULTS

**Vaginal exfoliative cytology:** The rats which were in estrous, exhibited entirely of cornified cells, characterized by larger cells with angular and irregularly shaped cytoplasmic borders. The mature cells were mostly non-nucleated cornified cells (Fig. 1). Some rats have exhibited signs of proestrous which was characterized by round nucleated epithelial cells and a few epithelial cells in early stages of cornification. A low incidence and degree of cornification at pro-estrus was normal.

**Clinical observations:** There was no gross evidence of acute toxicity after the administration of NMU. In group B, ten animals (mortality-22.22%) died during the experimental period. Post mortem examination and histopathological observations in those rats showed no specific changes. Some rats in group B developed conjunctivitis, dermatitis, severe cataract and respiratory infections. The rats from group B showed significant reduction in body weight after appearance of palpable tumour nodules. After 18th week, rats exhibited sharp decline in body weight.

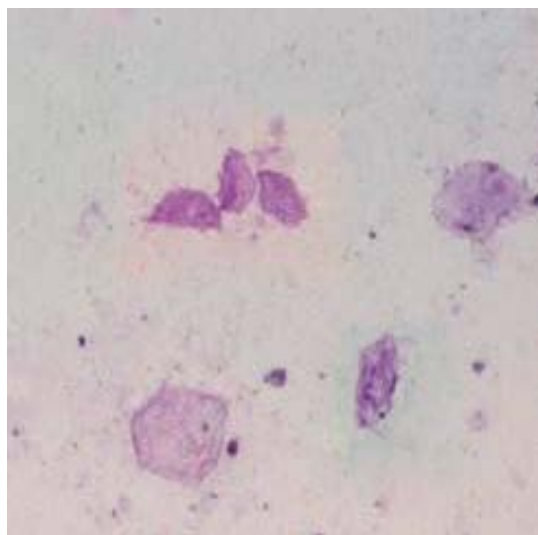


Fig. 1: Vaginal exfoliative cytology showing large, angular and irregularly shaped, non-nucleated, cornified (keratinised) cells characteristic of oestrus (Giemsa×200)

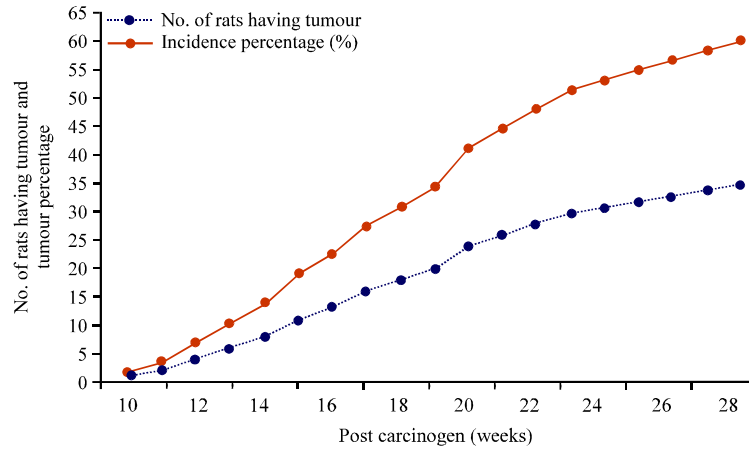


Fig. 2: Cumulative numbers of palpable tumours and its percentage as a function of time post carcinogen

Table 1: Tumour induction, tumour frequency, average latency period and tumour growth rate in NMU treatment group (Mean±SEM)

Parameters	Values
Tumour volume (cm <sup>3</sup> )	69±8.8
Tumour induction (DPI)	70
Average latency period (days)	107±4.1 (1st tumour-70 days)
Tumour frequency animal <sup>-1</sup>	4.7±0.33
Tumour incidence (%)	82.86
Tumour growth rate (cm day <sup>-1</sup> )	0.1
Average tumour weight at the time of euthanasia (g)	31±0.98

**Tumour induction, tumour frequency, latency period and incidence:** The first palpable tumour appeared on 70th day post carcinogen injection at the right 4th mammary gland. Subsequently, most of the rats developed tumours around 18-20th week in group-B. The observations recorded on palpation of mammary glands are presented in Fig. 2. The average growth rate of tumours was found to be 0.1 cm day<sup>-1</sup>. During 28 weeks of experimental period, totally 29 animals (out of 35) developed mammary tumours in group B. The incidence of tumours was 82.86% and maximum incidence of new tumours was found at 18th week of post carcinogen administration. The average number of tumours per animal (tumour frequency) was found to be 4.7±0.33. The average latency period was 107±4.1 days (1st tumour-70 days). The average tumour volume in cm<sup>3</sup> was found to be 69±8.8 and the average tumour weight in grams was found to be 31±0.98 (Table 1). The size of the tumours ranged from 4.5-8.0 cm in length and weight of the tumours ranged from 15-35 g.

Out of the 44 total numbers, 50% (22/44) appeared on the right mammary gland chain and 50% (22/44) occurred on the left mammary gland chain. Region wise, 81.82% (36/44) of the tumours appeared on abdominal-inguinal mammary glands (L4, L5, L6 and R4, R5, R6) and 18.18% (8/44) appeared on the cervical thoracic mammary glands (L1, L2, L3 and R1, R2, R3).

**Gross pathology:** Rats treated with NMU carcinogen did not show any gross tumour development in the initial 9 weeks but on 10th week, small, hard and nodular growth having 1 cm diameter was seen and it was growing fast. It had grown into a globular sized mass of 3.85 cm at 90 days and



Fig. 3: Rat showing large, multi-lobulated solid mass of tumour and invading into adjacent R4, R5, R6, L4, L5 and L6 mammary glands

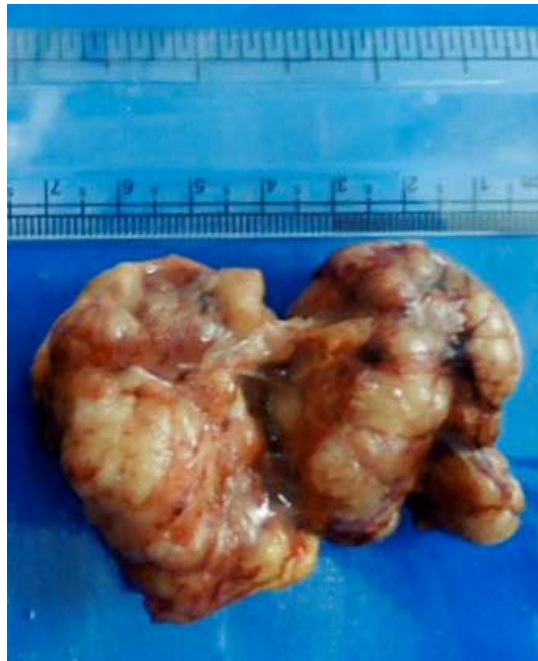


Fig. 4: Excised tumour from tumour bearing rat: Large, greyish white, multi-lobulated different sized tumour and measuring about 7.0 cm in length

further progressed to lemon-sized mass of 4.8×3.2 cm at 120 days post carcinogen administration. It further progressed into large, multi-lobulated solid mass of 7.6×5.2 cm invading into adjacent R4, R5, R6, L5 and L6 mammary gland at 150 days post carcinogen injection (Fig. 3 and 4). Some animals showed multiple tumorous growth which later on coalesced to become large mass of tumours (Fig. 3 and 4). One animal developed six tumours (R4, R5, R6, L4, L5 and L6), two



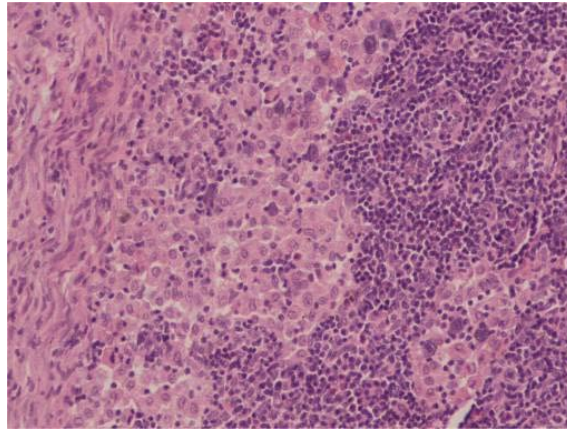


Fig. 5: Metastatic foci of neoplastic epithelial cells in the mammary lymph node (H and E×200)

animals developed five tumours each (R4, R5, L4, L5 and L6) and three animals developed four tumours each (L5, L6, R5 and R6). Three rats showed necrosis of the skin followed by sloughing of the skin and ulcer formation. The ulcer became infected and it emitted foul smell. The size and weight of the tumours varied greatly.

On post mortem examination, one rat showed tumour in the cervix which was large, white and hard in consistency. One rat showed small and hard nodule in the small intestine. Some rats showed enlarged spleen. There was also congestion, consolidation and pneumonia of lungs in some animals.

**Histopathology:** A total of 44 mammary tumours were diagnosed, in which 88.64% (39/44) were malignant and 11.36% (5/44) were benign. Among the malignant tumours, 33.33% (13/39) were non-invasive and 66.67% (26/39) were invasive. In this 2 (4.55%) tubular adenoma, 2 (4.55%) fibroadenoma, 1 (2.27%) papillary adenoma, 9 (20.46%) *in situ* solid cribriform carcinoma, 8 (18.18%) invasive solid cribriform carcinoma, 3 (6.82%) *in situ* papillary carcinoma, 6 (13.64%) invasive papillary carcinoma, 8 (18.18%) invasive tubular adenocarcinoma, 1 (2.27%) *in situ* comedocarcinoma and 3 (6.82%) invasive comedocarcinoma were diagnosed. Evidence of metastatic lesions was noticed in lungs and lymph node (Fig. 5). Lymphoid hyperplasia in spleen was observed in tumour bearing animals. The mammary tumours were classified according to Russo and Russo (2000) (Table 2).

**Tubular adenoma:** The tumour sections revealed proliferation of ductal and alveolar structures arranged in clusters and separated by moderate amount of connective tissue. Increase in the number of individual alveoli lined by a single layer of low cuboidal epithelial cells was observed (Fig. 6). Mitotic figures were rare ( $1.72 \pm 0.42$ /hpf).

**Fibro-adenoma:** The sections revealed lobular, alveolar and ductal structures surrounded by layers of fibrous tissue composed of immature fibroblasts with plumpy nucleus and mature fibrocytes with extensive deposition of collagen. The glandular structure was lined by single layer of low cuboidal epithelium and maintained the same relation with myoepithelium and basement

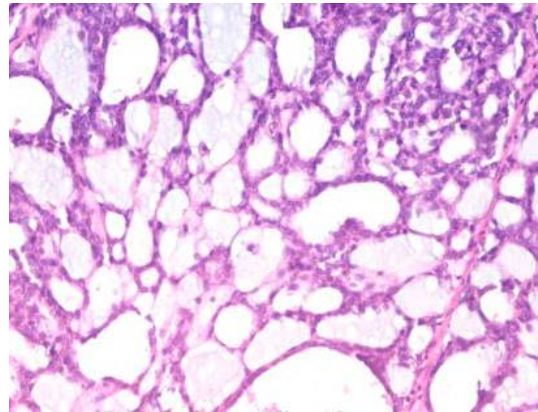


Fig. 6: Tubular adenoma showing proliferating ductal and alveolar structures arranged in clusters and lined by single layer of low cuboidal epithelial cells, separated by scant connective tissue (H and E×200)

Table 2: Histological classification of both mammary and non-mammary tumours induced by N-Methyl-N-Nitrosourea (NMU)

Tumour type	No. of tumours
Intra ductal proliferations (hyperplasia)	6
Tubular adenoma	2
Fibroadenoma	2
Papillary adenoma	1
Insitu solid cribriform carcinoma	9
Invasive solid cribriform carcinoma	8
Insitu papillary carcinoma	3
Invasive papillary carcinoma	6
Invasive tubular adenocarcinoma	8
Insitu comedocarcinoma	1
Invasive comedocarcinoma	4
Tricholemmoma	5
Papilloma of skin	2
Squamous cell carcinoma of skin	1
Leiomyo sarcoma of cervix	2
Adenocarcinoma of kidney	1

membrane as in normal mammary gland. The ducts and alveoli were constricted and distorted resulting in irregular alveoli with narrow lumen (Fig. 7). Mitotic figures were quite rare ( $1.60 \pm 0.58/\text{hpf}$ ).

**Papillary adenoma:** Microscopic examination of the tumour section revealed presence of finger like papillary structures growing inwards into the luminal space. Papillae were lined by proliferating neoplastic epithelial cells with central connective tissue core. The lining neoplastic cells were arranged in single layer. Mitotic figures were few ( $1.68 \pm 0.0/\text{hpf}$ ).

**In situ solid cribriform carcinoma (DCIS):** The sections showed proliferating neoplastic cells in lobules arranged in solid sheets with frequent formation of round or irregular shaped secondary

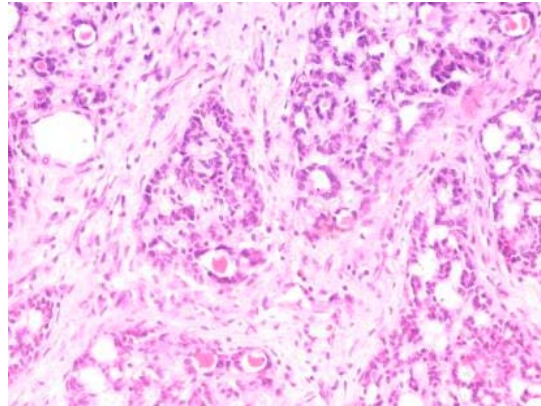


Fig. 7: Fibroadenoma showing constricted and distorted alveoli with distinct myoepithelial cells surrounded by thick layer of connective tissue (H and E×200)

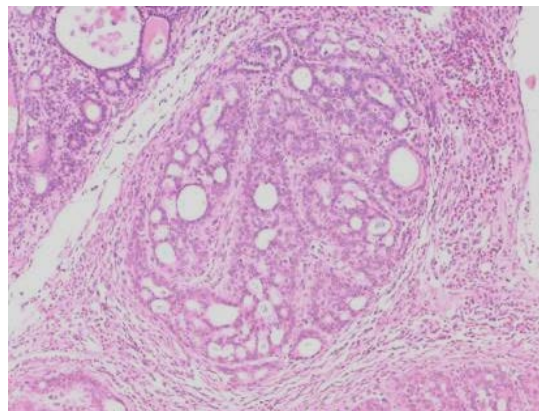


Fig. 8: *In situ* solid cribriform carcinoma (DCIS): Solid sheets of neoplastic cells with irregular shaped secondary lumina within the lobule showing sieve like pattern with intact basement membrane (H and E×200)

lumina, giving an appearance of sieve. The cell proliferation was confined within the lumen of the duct with basement membrane remaining intact (Fig. 8). Mitotic figures were moderate ( $3.01 \pm 0.61/\text{hpf}$ ).

**Invasive solid cribriform carcinoma:** The tumour sections revealed proliferation of neoplastic cells in lobules arranged in solid sheets with frequent formation of spherical or irregular shaped secondary lumina of variable sizes, giving an appearance of sieve (Fig. 9). Neoplastic cells evinced aggressive growth with marked pleomorphism. The neoplastic cells were seen invading into the surrounding stroma. The invading neoplastic cells were surrounded by fibro-vascular connective tissue exhibiting mild desmoplastic reaction which was heavily infiltrated with neutrophils, mononuclear cells and mast cells. Mitotic figures were numerous ( $5.2 \pm 0.39/\text{hpf}$ ). In three cases, the tumour invaded into adjacent inguinal lymph node (Fig. 5).

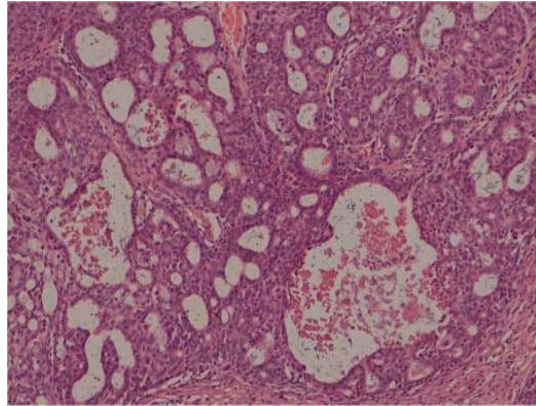


Fig. 9: Invasive solid cribriform carcinoma: Invasive tumour cells arranged in solid sheets, with numerous irregular secondary lumina and pleomorphic neoplastic cells with numerous mitotic figures (H and E×200)

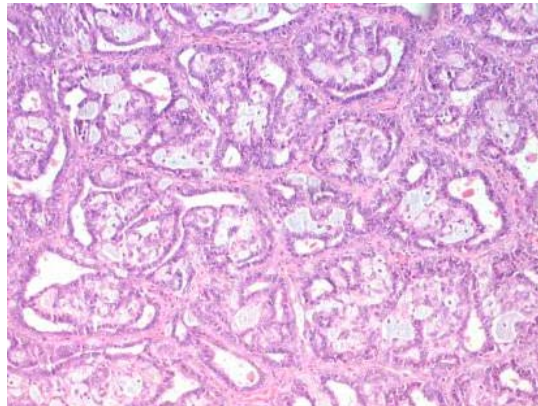


Fig. 10: *In situ* papillary carcinoma (DCIS): Papillary projections into the lumen of the dilated ducts and alveoli with intact basement membrane and very little fibro-vascular core (H and E×100)

***In situ* papillary carcinoma (DCIS):** The sections revealed proliferation of neoplastic epithelial cells growing inward in the lumen to form finger like papillary structures with very little fibro-vascular core, confined within the lobule with intact basement membrane (Fig. 10). Mitotic figures were moderate ( $3.21 \pm 0.04/\text{hpf}$ ).

**Invasive papillary carcinoma:** The tumour sections showed proliferating neoplastic cells arranged in long finger like projections with a central delicate fibro-vascular core (Fig. 11) and exhibited features of severely malignant. These lesions were classified into grade I or grade II depending upon epithelial thickness and cytological characteristics.

**Grade II:** Neoplastic cells were arranged in five to ten layers thickness with long finger like papillae projected into the lumen over sparse fibro-vascular core. Secondary projections (papillae)

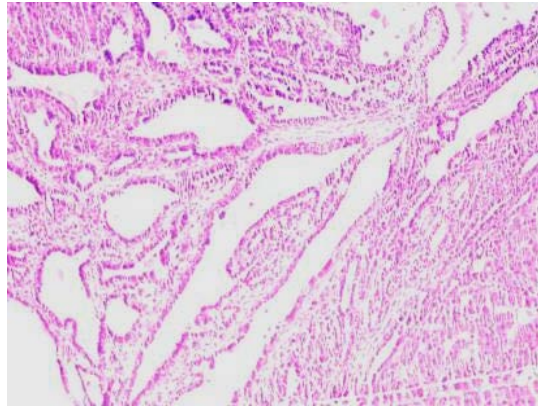


Fig. 11: Invasive papillary carcinoma: Numerous papillary projections sustained by thin connective tissue core (H and E×100)

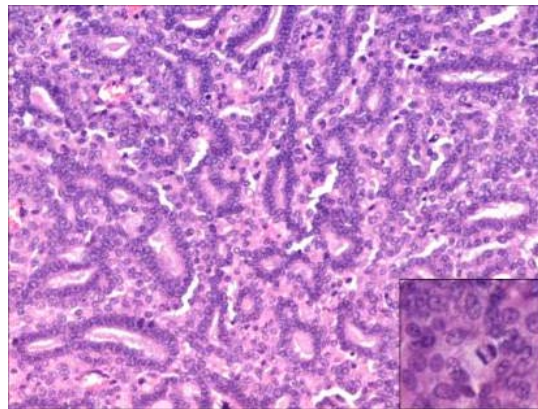


Fig. 12: Invasive tubular adenocarcinoma: Ductular and alveolar structures arranged in clusters separated by scarce amount of connective tissue. H and E×200. Inset showing highly pleomorphic neoplastic cells with numerous mitotic figures (H and E×400)

were formed by solid cluster of neoplastic epithelial cells with serrated luminal border (Fig. 11). Papillae from one lobule were found entered into adjacent lobule by penetrating basement membrane. The neoplastic cells had vesicular type of nucleus with prominent nucleoli. Mitotic figures were prominent ( $5.4 \pm 0.18/\text{hpf}$ ).

**Invasive tubular adenocarcinoma:** The tumour sections showed proliferation of ductal or alveolar structures arranged in clusters and separated by scarce amount of connective tissue. The neoplastic cells contained variable sized, round to oval enlarged nuclei with prominent nucleoli (Fig. 12). Mitotic figures were present in high number ( $5.6 \pm 0.24/\text{hpf}$ ).

**In situ comedocarcinoma (DCIS):** It was characterized by distended ductal structures lined by a multi-layered epithelium and centrally located necrotic debris (Fig. 13). The involved ducts became distorted depending upon the extent of growth. The stroma surrounding each individual

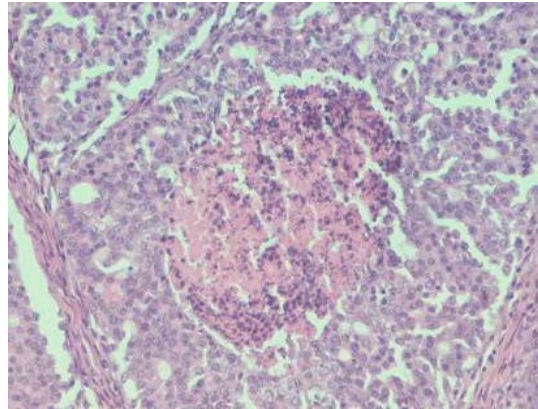


Fig. 13: *In situ* solid comedocarcinoma (DCIS) showing distended ductal structures lined by a multi-layered epithelium and centrally located necrotic debris with intact basement membrane (H and E×200)

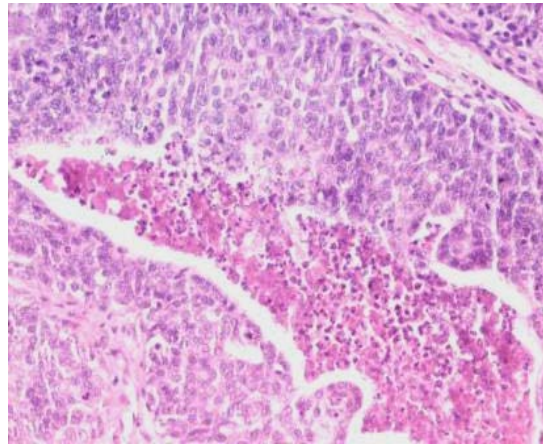


Fig. 14: Invasive comedocarcinoma showing distended ductal structures lined by a multi-layered epithelium with centrally located necrotic debris (H and E×200)

ductal structure was composed of dense fibrous tissue and exhibited a marked desmoplastic reaction. Mitotic figures were moderate ( $1.95 \pm 0.71/\text{hpf}$ ).

**Invasive comedo-carcinoma:** Invasive comedo-carcinoma was characterized by presence of distended ductal structures lined by a multi-layered epithelium and centrally located necrotic debris (Fig. 14). The stroma surrounding the individual ducts exhibited marked desmoplastic reaction. Individual neoplastic cells were highly pleomorphic with large hyperchromatic nuclei containing prominent nucleoli. Mitotic figures were moderate ( $4.1 \pm 0.0/\text{hpf}$ ).

**Intra-ductal proliferation (IDP) or hyperplasia:** The earliest changes observed in the mammary parenchyma after carcinogen treatment of virgin rats were intra-ductal proliferation or

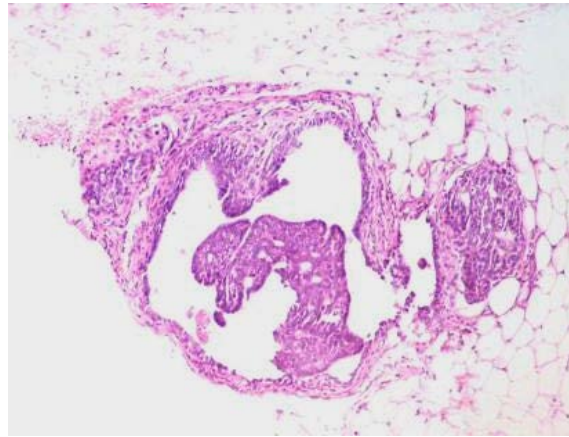


Fig. 15: Intra-ductal proliferation or hyperplasia resulting from dilatation of terminal end buds and thickening of the lining epithelium by two or more layers (H and E×200)

hyperplasia resulting in dilatation of terminal end buds and thickening of the lining epithelium by two or more layers (Fig. 15). Depending on the degree of thickening, circumference of the acini and ducts, position and size of the primary lumen, degree of desmoplastic reaction and changes in the lining epithelium, the lesions were further classified in to mild, moderate and florid (severe) hyperplasia.

**Moderate hyperplasia:** These lesions featured more than four layers of ductal lining epithelial cells and in few cases, a broad bridge was noticed across the lumen. Mild desmoplastic reaction was also observed around the acini and ducts (Fig. 15).

**Immunohistochemical expression of PCNA, VEGF and PECAM-1:** The benign tumours like tubular adenoma, fibroadenoma and papillary adenoma showed weak immunostaining for the proliferation marker PCNA -  $34.56 \pm 1.37$ ,  $23.73 \pm 2.75$  and  $38.12$ , respectively. All the three benign tumours were negative for VEGF and PECAM-1 immunolabeling. The malignant tumours exhibited strong and intense nuclear immunostaining for the proliferation marker PCNA and strong cytoplasmic immunolabelling for both the angiogenic markers VEGF and PECAM-1/CD-31. *In situ* solid cribriform carcinoma exhibited average PCNA, VEGF and PECAM-1 expression of  $77.2 \pm 0.07$ ,  $12.9 \pm 0.65$  and  $13.75 \pm 0.34$ , respectively. Invasive tubular carcinoma exhibited average PCNA, VEGF and PECAM-1 expression of  $80.9 \pm 1.8$  (Fig. 16),  $17.8 \pm 1.2$  (Fig. 17) and  $16.4 \pm 0.56$ , respectively. Invasive solid cribriform carcinoma exhibited highest PCNA ( $81.6 \pm 1.1$ ). The expression pattern ranged from 48.7 to 81.6% for PCNA. Both the epithelial and connective tissue components of the tumour showed positivity for proliferation marker PCNA. The average VEGF and PECAM-1 expression was  $16.4 \pm 0.20$  and  $13.4 \pm 0.85$  (Fig. 18), respectively. Invasive papillary carcinoma exhibited average PCNA, VEGF and PECAM-1 expression of  $68.8 \pm 1.9$ ,  $19.5 \pm 0.89$  and  $14.3 \pm 1.9$ , respectively. The neoplastic cells revealed noticeable variation in the staining pattern of the proliferation marker ranging from 42.8-68.8%. *In situ* papillary carcinoma exhibited average PCNA, VEGF and PECAM-1 expression of  $64.3 \pm 0.41$ ,  $15.4 \pm 0.05$  and  $17.8 \pm 0.62$ , respectively.

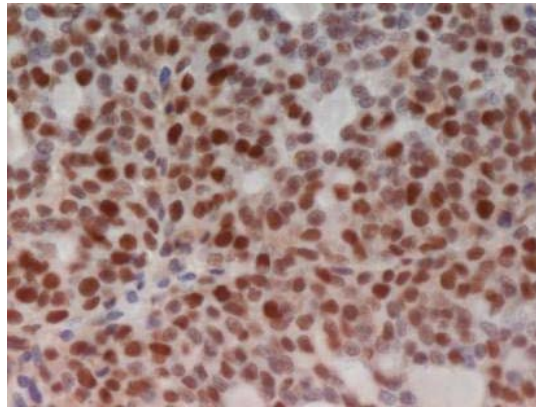


Fig. 16: Invasive tubular adenocarcinoma: Neoplastic epithelial cells exhibiting strong PCNA-positive immunolabelling (IP-DAB-MH×400)

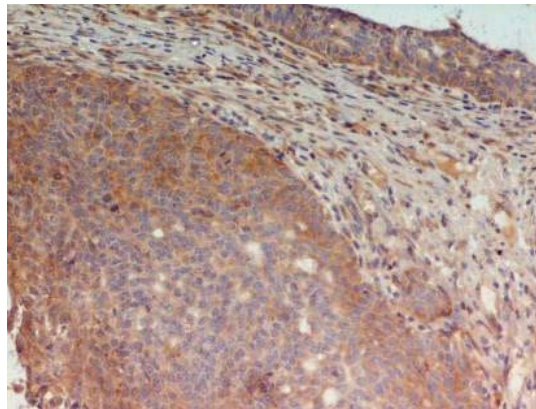


Fig. 17: Invasive tubular adenocarcinoma: Tumour cells showing strong diffuse cytoplasmic expression of VEGF protein (IP-DAB-MH×200)

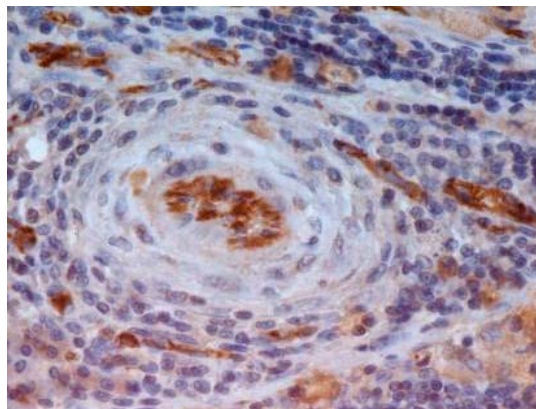


Fig. 18: Invasive solid cribriform carcinoma: Immunostaining of PECAM-1 in the tumour blood vessels and tumour cells exhibiting strong microvessel density (MVD) (IP-DAB-MH×400)



## DISCUSSION

Studies pertaining to experimental animal models have demonstrated that mammary cancer is a complex multistep process that can be induced either by chemicals, radiation, viruses or genetic factors. Cells of rodent origin are much easier to be transformed by chemical carcinogens, owing to less efficient DNA repair, poorer control of genetic stability and altered control of gene expression through processes such as DNA methylation (Thompson *et al.*, 1995; Masso-Welch *et al.*, 2000; Russo and Russo, 2000; Kim *et al.*, 2004). The NMU model has several advantages, such as reliability of tumour induction organ site specificity, tumour of ductal origin and predominantly carcinomatous histopathologic characterization and the ability to examine tumour initiation and promotion processes (Thompson and Adlakha, 1991).

In the present study, rats were administered with NMU carcinogen after confirming oestrus by vaginal exfoliative cytology. The oestrus identification at the time of NMU carcinogen injection was found to be the simplest method for inducing more number of experimental mammary tumors in rats. Rivera *et al.* (1994) reported that the tumours induced by this method are oestrogen-dependent. Tumour incidence was significantly higher in oestrus (95.2%) than proestrus (71.4%) and diestrus (77.4%). Mean number of tumours per animal was high in oestrus ( $4.4 \pm 3.2$ ), proestrus ( $3.8 \pm 3.6$ ) and diestrus ( $3.2 \pm 1.8$ ). Lindsey *et al.* (1981) reported average latency period for tumour appearance in diestrus, proestrus and estrus groups were 104.4, 83.6 and 91.4 days, respectively, following the first NMU injection. The mean number of tumours per rat was significantly higher in rats injected on proestrus (4.5) or estrus (4.3) than on diestrus (2.0). Carrera *et al.* (2005) also injected NMU to the rats which were in estrus and at the first NMU injection verified tumours by daily vaginal smears.

In the present investigation, NMU carcinogen at the dose rate of 50 mg kg<sup>-1</sup> body weight intra-peritoneally was administered to female Sprague Dawley rats at 50, 80 and 110 days of age. Susceptibility of the mammary glands to the carcinogen depends on the age of animals. High susceptibility of female rats can be observed when the carcinogen is administered during the postnatal period, between 40 to 60 days of early puberty with highly proliferating Terminal End Buds (TEB) in the mammary gland which are the targets for chemical carcinogens (Huggins *et al.*, 1959).

During 28th weeks of experimental period, tumour incidence was found to be 82.86% (29/35) which was significantly high when compared to the tumour incidence of 73% observed by Gullino *et al.* (1975), 76% by Liska *et al.* (2000), 55% by Yuri *et al.* (2005), 45% by Vegh and de Salamanca (2007). However, it was low when compared with 83% by Moon *et al.* (1977), 90% by Thompson and Meeker (1983) and 100% by Thompson and Adlakha (1991).

In the present study, first palpable tumour was noticed on 70 days of post carcinogen administration and the average latency period was found to be  $107 \pm 4.1$  days which was higher when compared to the observations of 86 days by Gullino *et al.* (1975) and 42 days by Thompson and Adlakha (1991) but significantly lower when compared to  $113 \pm 4.2$  days by Carrera *et al.* (2005), 90-120 days by Vegh and de Salamanca (2007) and 115 days by Mayilkumar (2009). The average number of tumours per animal (tumour frequency) was found to be  $4.7 \pm 0.33$  tumours which was low when compared to 5.3 rat<sup>-1</sup> reported by Thompson and Adlakha (1991) and high compared to 2.71 rat<sup>-1</sup> by Gal *et al.* (2008) and 2.25 rat<sup>-1</sup> by Mayilkumar (2009).

The average growth rate of tumours was found to be 0.1 cm per day, the average tumour volume in cm<sup>3</sup> was found to be  $69 \pm 8.8$  and the average tumour weight in grams was found to be

31±0.98. Body weight gain of experimental (with tumours and without tumours) and control animals compared at fortnightly intervals showed a significant ( $p < 0.05$ ) difference. The group-B rats showed significant reduction in body weight after appearance of palpable tumour nodules which were in agreement with earlier findings (Thompson and Adlakha, 1991; Liska *et al.*, 2000; Mayilkumar, 2009) wherein, the weight reduction in NMU treated groups was evident only after the tumours started appearing. The body weight decline was may be due to tumour burden. However, Perse *et al.* (2009) reported no significant change in body weight of both groups.

In the present study, equal numbers of tumours were developed in left and right mammary gland chain. These findings were in agreement with the previous observations of Thompson and Meeker (1983) and Thompson and Adlakha (1991), in which no significant difference was observed between left versus right mammary chain in the appearance of mammary tumours. Whereas, Mayilkumar (2009) reported incidence was more in left mammary chain when compared to the right. Out of all the diagnosed tumours, 81.82% (36/44) were produced in abdominal inguinal mammary glands and 18.18% (8/44) were developed in cervical thoracic mammary gland pairs which were 4.8 times higher. These findings were quite higher in comparison to the observations made by Thompson and Meeker (1983) and Thompson and Adlakha (1991) who recorded 2.4 and 2 times more tumours in abdominal and inguinal pairs, respectively. However, it was lower in comparison to the observations made by Mayilkumar (2009) who recorded 6.3 times more tumours in abdominal and inguinal mammary glands. Significant difference was observed in the occurrence of mutant ras 12 carcinomas between the cervical-thoracic and the abdominal-inguinal mammary glands as three times more carcinomas were mutant in the former as in the latter glands (Lu *et al.*, 1998).

A total of 44 mammary tumours were diagnosed, in which 88.64% (39/44) were malignant and 11.36% (5/44) were benign. Among the malignant tumours, 33.33% (13/39) were non-invasive and 66.67% (26/39) were invasive. These findings were slightly low when compared to 100% incidence of malignant tumours observed by Thompson and Adlakha (1991), 96.2% malignant by Vegh and de Salamanca (2007) and 91.77% malignant by Mayilkumar (2009). Some tumours were metastasized into mammary lymph node. These findings corresponded with the observations of McCormick *et al.* (1981) who suggested that getting metastasis to distant sites is one of the advantages in the NMU induced tumour model. Some authors have reported metastases to lungs, liver, spleen and bone marrow (Gullino *et al.*, 1975; McCormick *et al.*, 1981). However, in the present study, no metastasis occurred to lungs, spleen and liver which could be probably due to shorter observation period of about 28 weeks against 2 or 3 years or the whole life span of the animal when metastasis become fully evident as observed by Russo and Russo (2000). Absence of metastasis in chemically induced rodent tumours was also reported by Rose *et al.* (1980) and Chan *et al.* (2005).

For many years, pathologists have been using mitotic index as a tool for determining the aggressiveness of the tumour and to grade them for prognostic behaviour. The average value of mitotic index in NMU mammary tumours was found to be  $4.5 \pm 0.46$ /hpf. Elevated level of PCNA appears in the nucleus during the late G1 phase becomes maximal during the S-phase and declines again during the G2 phase and M phases (Bravo *et al.*, 1987). In the present study, the average value of PCNA in NMU induced mammary tumours was found to be  $77 \pm 2.6$  and expression intensity varied depending upon the extent and aggressiveness of the tumour. The elevated levels of PCNA were observed when compared to the observations of Aceves *et al.* (2009) and Mayilkumar (2009) reporting 50 and 66%, respectively. However, in contrary, Kang *et al.* (2004) observed no

significant difference between normal mammary gland (8.72±4.86) and in NMU induced mammary tumours (7.99±6.20). Higher PCNA index in invasive tumours was probably due to more proliferating cells in S-phase of cell cycle.

Expression of VEGF and PECAM-1 proteins was demonstrated for the first time in NMU induced rat tumours. Studies on tumour angiogenesis in nude mice indicate that VEGF/VPF expression is critical for effective tumourigenesis and tumour angiogenesis. In the present study, VEGF immunostaining was cytoplasmic and varied in intensity from 0.64 to 16.2%. The average value of VEGF and PECAM-1 in NMU induced mammary tumours was found to be 16.2±0.86 and 15±0.69, respectively. Expression of VEGF became more pronounced following carcinogenesis. There was no published report about VEGF expression and microvessel density evaluation using immunohistochemistry against PECAM-1 antigen, in NMU-induced rat mammary carcinogenesis. However, expression of VEGF became more pronounced following N-butyl-N-(4-hydroxybutyl) induced bladder carcinogenesis. In rat bladder carcinoma, tumours consisted of mixtures of areas with high expression of VEGF and areas with slight to no labelling (Wakui *et al.*, 1999). In the present study, the NMU control group showed more VEGF and PECAM-1 expression which was higher when compared to the observations of Rather (2012), who reported 3.1±0.37 and 5.5±1.92, respectively.

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