GSC Biological and Pharmaceutical Sciences
eISSN: 2581-3250 CODEN (USA): GBPSC2
Cross Ref DOI: 10.30574/gscbps
Journal homepage: https://gsconlinepress.com/journals/gscbps/

(RESEARCH ARTICLE)

Check for updates

Biochemical and histopathological evaluation of an *in vivo* model of breast cancer

Neha Sharma [1], Anila Negi [1], Dharambir Kashyap [2], Amanjit Bal [2] and Shalmoli Bhattacharyya [1,*]

[1] *Department of Biophysics, PGIMER, Chandigarh, India-160012.*
[2] *Department of Histopathology, PGIMER, Chandigarh, India-160012.*

GSC Biological and Pharmaceutical Sciences, 2021, 16(01), 202–210

Publication history: Received on 01 June 2021; revised on 26 July 2021; accepted on 28 July 2021

Article DOI: https://doi.org/10.30574/gscbps.2021.16.1.0193

Abstract

Though, the clinical management of breast cancer has improved significantly over the past 30 years, it still remains the leading cause of cancer-related female death worldwide. Prevention is the fundamental issue in breast cancer control, for which identification markers in terms of initiation and promotion are necessary. To understand this, an animal model which can recapitulate the early symptoms of breast cancer development and progression is required. Present study is an attempt to develop a convenient and economical *in-vivo* animal model of breast cancer suitable to conduct such study. Female Wistar and SD rats were injected with different doses and routes of administration of 7, 12-Dihydroxymethylbenz (a) anthracene (DMBA). Animals were observed for the presence of visible/palpable tumours in mammary glands. Various parameters (Tumor morphology, oxidative stress and histopathological studies were studied in different tissues (mammary, lungs, kidney, liver) after the appearance of mammary tumours in rats. After 14 weeks all the animals developed breast carcinomas. The results of this study revealed a significant difference in oxidative stress parameters between DMBA treated and control groups and these alterations were strain dependent. The H&E staining of mice mammary tissue showed development of metaplastic triple negative breast cancer. Immunohistochemistry observation confirmed the triple negative nature of mammary tumours developed in the mice. Data confirmed that DMBA can be used as breast cancer initiator and present model can be further exploited to screen potential anti-breast cancer compounds *in vivo*.

Keywords: Animal model; Breast cancer; DMBA; Oxidative stress; Triple negative breast cancer

1. Introduction

Breast cancer (BC) is one of the most frequent malignancy and second leading cause of cancer death among women worldwide. Recently more attention has been directed towards its prevention. Despite development of new treating strategies and advances in identifying novel targeted therapies the affected number is on rise [1]. It can be defined as an uncontrolled growth of breast cells originating from breast tissue. It could be as a result of mutations in the genes responsible for regulating the growth of cells [1]. Numerous factors, including molecular and cellular pathways could be involved in the initiation and progression of breast cancer. Classification of clinical subtypes (ER+, PR+, HER2+, and TNBC (Triple-negative) increases the complexity of breast cancers, which thus necessitates further investigation [2]. Diagnosis of breast cancer at an early stage by available screening methods is based on analyzing anatomical changes of the breast. Hence, it may take years for the tumour to grow to a sufficient size to be detectable [3]. Therefore, an experimental system is needed that closely mimics the human disease, and elucidate the influence of host factors on the initiation of the neoplastic process and to discover whether it can be manipulated by treatment of the host.

For translation of anticancer therapies and imaging agents that have shown promising results in invitro studies, the development of an invivo model that can recapitulate the hallmark clinical symptoms of breast cancer is necessary.

* Corresponding author: Shalmoli Bhattacharyya
Department of Biophysics, PGIMER, Chandigarh, India-160012.

Mouse models used in breast cancer research provide an essential approach to examine the mechanisms and genetic pathway in cancer progression and metastasis [4].

There are various methods of inducing breast cancer in animal models by the use of chemical compounds, transgenic animals, ionizing radiation, and tumour cell transplantation. Tumour induction by chemical compounds usually has advantages such as easy to use and controllable as well as disadvantages such as high toxicity to humans, tissue constraints and tumours in other tissues. The use of ionizing radiation is also dangerous, and its benefits can be accelerated by induction of tumour, low cost and easy to use. Other methods include the transplantation of cultured cells and transgenic animals, in which there are no hazards of prior methods, but there are some disadvantages such as their time and cost [5]. The interpretation and extrapolation of data derived from these models to humans, offers scope for translational medical research in early-stage clinical trials. Therefore, these play important role in improving the current clinical attrition rates for novel oncology therapeutic agents.

Commonly used chemical carcinogen-based models are N-Methyl-N-Nitrosourea (NMU), 7, 12-dimethylbenzanthracene (DMBA), methylchloranthracene (MCA), diethylnitrosoamine (DEN) and azoxymethane (AOM) [6]. DMBA and NMU induced rat mammary carcinoma models are widely used to study the human breast cancer [7]. The chemically induced models offer higher cost-effectiveness and greater ease of application. Further, keeping in view of current side effects of available chemo-preventive drugs i.e., Tamoxifen, Raloxifene, Aromatase inhibitor (exemestane, letrozole and anastrozole) and Faslodex etc more research should be focused on the new drugs with high specificity, low mortality rate and lesser side effects [8].

In order to study the pertinent questions in cancer research *in vivo* models of the disease serve as important research tools. The intention is to employ organ specific animal models to determine which agents are likely to be helpful in preventing specific forms of cancer.

Therefore, present study is an attempt to design a chemically induced animal model of breast cancer which can closely mimic the human disease.

2. Material and methods

Animals: Healthy Female Wister and SD strain rats weighing 300-350g (Age 45 to 60 days) were obtained from animal house of PGIMER. They were divided into different experimental groups. The animals were kept in polyproplylene cages under hygienic conditions and were provided standard animal feed and water *ad libitum* throughout the experiment. All procedures were done as per ethical guidelines.

Group I (n = 5) Wistar rats served as control and were sacrificed along with other rats.
Group II (n = 5) SD rats served as control and were sacrificed along with other rats.
Group III (n = 5) Wistar rats were injected with 15mg/kg b.wt DMBA (i.p) and were sacrificed 14 weeks after initiation of treatment.
Group IV (n = 5) Wistar rats were given 20mg/kg b.wt DMBA (p.o) and were sacrificed 14 weeks after initiation of treatment.
Group V (n = 5) SD rats were injected with 15mg/kg b.wt DMBA (i.p) and were sacrificed 14 weeks after initiation of treatment.
Group VI (n = 5) SD rats were given 20mg/kg b.wt DMBA (p.o) and were sacrificed 14 weeks after initiation of treatment.

2.1. Breast Cancer Induction by Chemical Carcinogen:

7, 12-dimethylbenz (a) anthracene (DMBA) was dissolved in 0.5 ml sunflower/corn oil and 0.5ml saline for the induction of breast cancer in each female rat. A single dose injection of DMBA (15 mg/kg body weight) was injected intraperitonially (i.p) into the rats in test group III & V. A single dose of DMBA (20 mg/kg body weight) was given intragastrically (p.o) to the rats in test group IV & VI. In the control group I and II animals were given sunflower oil (0.5 ml) and 0.5ml of saline. Both groups were kept under prescribed conditions and observed and palpated weekly to determine the development, localization and size of tumour. Animals were weighed monthly. Fourteen weeks after DMBA administration rats were anesthetized using intramuscular injection of ketamine (40–87 mg/kg) and xylazine (5–13 mg/kg) and sacrificed by cervical decapitation. All breast tissues were resected, washed in 0.9 % NaCl and frozen rapidly at -80 °C. After autopsy mammary tumours were measured and findings were recorded.

All experiments were done with written approval from the Institute animal ethics committee and all guidelines for maintenance and use of animals were followed.

2.1.1. Protein estimation

Protein estimation was done by the method of Lowry et al [9] in homogenate as well as post mitochondrial fraction (PMF) obtained after homogenization of various tissue samples in Phosphate buffered saline (PBS).

2.1.2. Reduced Glutathione (GSH)

Method described by Ellman [10] was followed to estimate the reduced glutathione levels in the tissue. Accordimg to this method 5, 5-dithiobis2-nitrobenzoic acid (DTNB) is reduced by –SH groups leads to the formation of 2-nitro-5-mercaptobenzoic acid per mole of SH. The release of nitro mercaptobenzoic acid imparts yellow color to the reaction mixture which can be used to measure –SH groups at 412 nm.

2.1.3. Lipid Peroxidation

Levels of malondialdehyde (MDA), which is a degradation product of peroxidised lipids serves as an index for determining the extent of lipid peroxidation from breakdown of polyunsaturated fatty acids. MDA levels were determined in the Post mitochondrial fraction (PMF) by following the method described by Wills [11].

2.1.4. Reactive Oxygen Species (ROS)

DCFH-DA which is oxidized into fluorescent DCF by different intracellular ROS was used to estimate total ROS production. According to the method of Best [12] samples were homogenized in phosphate buffer (0.1 M, pH 7.4) and incubated with 12ul of DCFH-DA (1.25 mM stock solution in methanol) at 37'C for 30 min in the dark. Perkin Elmer fluorescent spectrometer at an excitation wavelength of 488 nm with emission filter set at 525 nm. Was used to read the fluorescence of samples.

2.1.5. Histopathological Study

Rats were sacrificed and tissues (tumours, breast, kidney, liver and lungs) were removed. After removal tissues were immediately fixed in 10% formalin fixative for about 24-48 h. After fixation, tissues were dehydrated in ascending grade of alcohol, embedded in wax following standard technique. 5–7-micron thick sections were cut and spread on glass slides. Slides were subjected to hematoxylin and eosin staining, mounted in DPX, and viewed under light microscope.

2.1.6. Immunohistochemistry (IHC)

The immunohistochemistry for molecular subtyping of developed mammary tumour was performed on the formalin-fixed and paraffin-embedded (FFPE) tissue sections using anti-bodies.

ER, PR and HER-2. In brief, 5-µm sections were obtained using a microtome, transferred onto poly-a-lysin coated slides, and dried at 60°C for 30 min. After incubation for 1h with the primary antibodies, immunodetection was performed using biotinylated anti-mouse immunoglobulin, followed by peroxidase-labelled streptavidin using a labelled streptavidin biotin kit with 3, 3′- diaminobenzidine (DAB) chromogen as the substrate. Haematoxylin was used as counter staining against nuclei.

2.1.7. Statistical analysis

The results are expressed as mean ± SD of five animals in each group. For statistical significance, the data were analyzed using one-way ANOVA followed by post hoc test.

3. Results

3.1. Gross examination

The gross examination of the breast tumours in control and experimental group of rats was done every month. DMBA-induced group III and IV rats showed tumour mass present in mammary gland of lower abdominal right flank (Figure 1) at week 14 following DMBA administrtation. While, there was no visible/palpable tumour observed in the mammary gland of control groups 1 and II (SD and Wistar) and DMBA treated SD rats groups V and VI. Therefore, for further

studies tumours from both groups III and group IV were used. Tumour sizes were measured using Vernier caliper with 0.1 mm graduation. Tumour incidence = % no. of animals having tumours was also calculated (Table 1).

3.1.1. Legends

Table 1 Indicates route of administration of DMBA, tumour incidence and tumour volume in different groups

Groups (n=5)	**Dose (mg/ml)**	**Route of administration**	**Tumour incidence**	**Tumour measurement (lxbxh)**
I Control (Wistar)	Sunflower oil+saline	i.p , p.o	-	-
II Control (SD)	Sunflower oil+saline	i.p, p.o	-	-
III (Wistar)	15	i.p	0.6	120mm^3 (approx..)
IV (Wistar)	20	p.o	0.5	100mm^3 (approx..)
V (SD)	15	i.p	-	-
VI (SD)	20	p.o	-	-

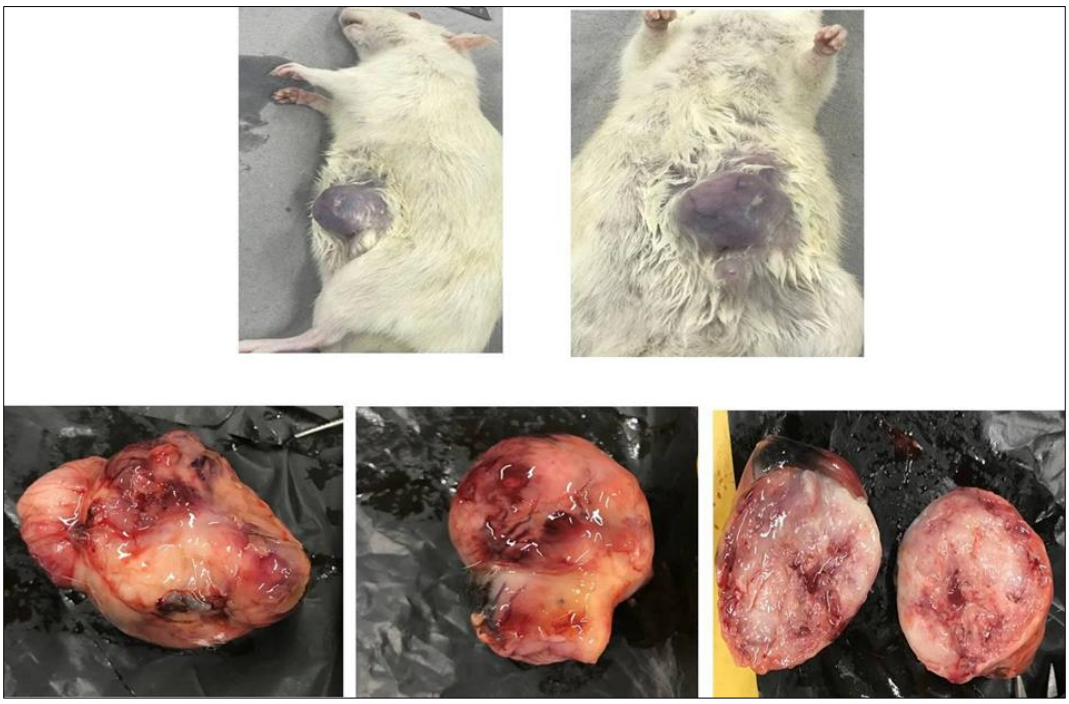

Figure 1 Gross examination of breast tumours as observed in DMBA treated group (Group III and IV)

3.2. LPO, ROS and GSH levels

Following DMBA injection at week 14, a significant ($p \leq 0.001$) elevation in MDA levels were observed in tissues from different organs (breast, kidney, liver and lungs) of rats as compared to control group (Figure 2). Similarly, a significant ($p \leq 0.001$) increase in total ROS production was observed in tissue from different organs (breast, kidney, liver and lungs) of DMBA treated rats control rats (Figure 2). Reduced glutathione levels (GSH) were found to be significantly ($p \leq 0.001$) reduced in tissue from different organs (breast, kidney, liver and lungs) of DMBA treated rats w.r.t control rats (Figure 2). Our results are in line with the results obtained by Batcioglu et al[13]; Mani et al[14] in DMBA induced rat model of breast cancer.

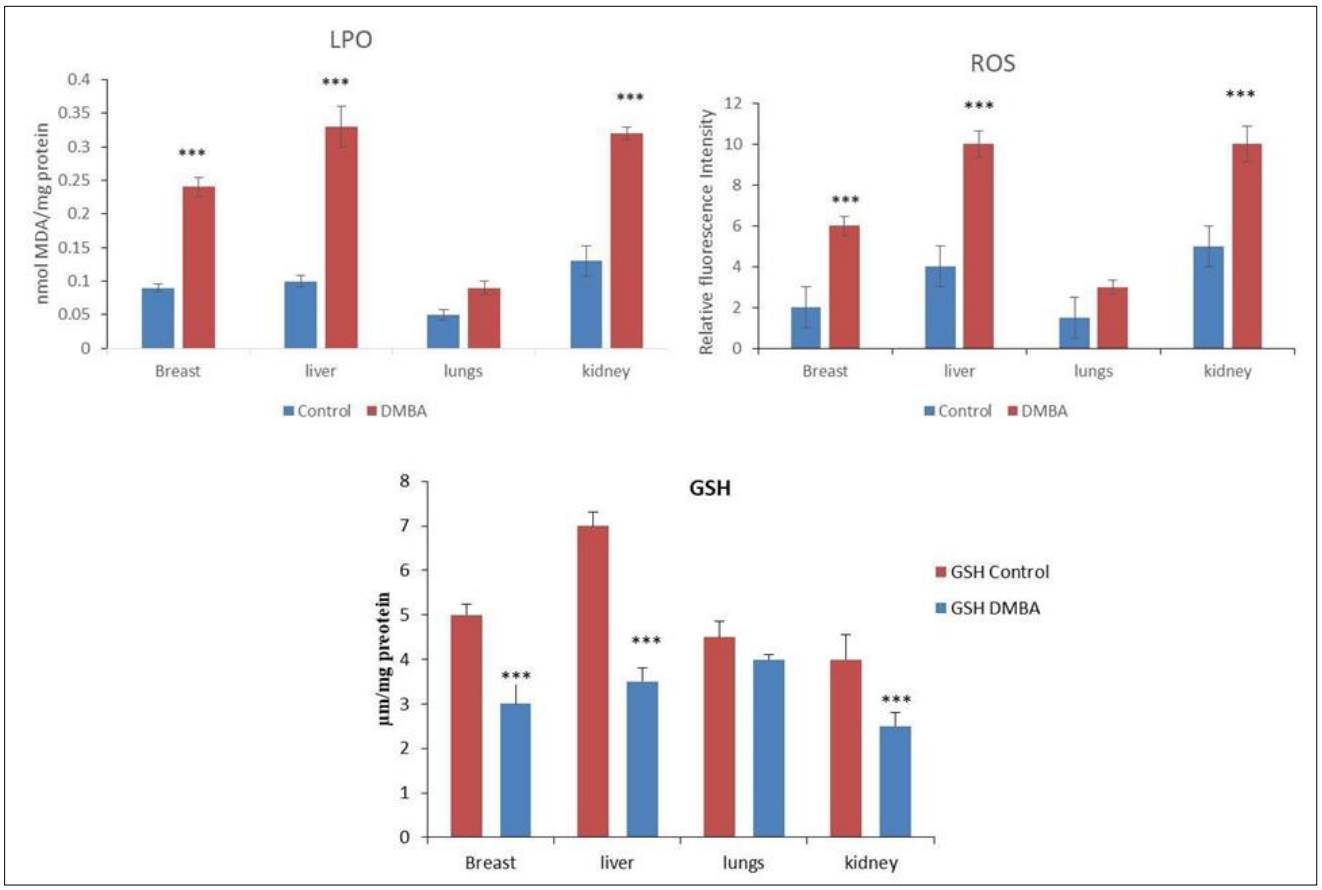

Figure 2 Effect of DMBA on the levels of biochemical parameters (a) Lipid peroxidation (MDA levels) (b) Total ROS production (c) Reduced glutathione levels (GSH) in different organs (Liver, Kidney, Lungs and breast tumour tissue). Values are expressed as mean ± SD (n=5 rats/each group). Statistical significance was set at $p<0.05$ compared with control

3.3. Histopathology

Histopathologic studies were done in control and experimental groups. Hematoxylin & eosin and immunostaining results for surrogate markers (ER/PR/HER2+) were assessed by pathologist using light microscopy. DMBA treated rat mammary tissue morphologically showed transformed tumour cells as compared to the control. Tumour cells had spindled morphology giving the characteristic of spindle cell metaplastic carcinoma. The tumour stroma showed infiltration of lymphocytes (Figure 3b&3c: Lymphocytes are marked in the slides in circular shape whereas spindle shaped cells were seen in oval marking).

For assessment of ER/PR staining, nuclei positivity of ≥1% in tumour cells was used to define ER and PR positivity. Further, HER2+ staining was analyzed following categories: 0 = no immunostaining; 1+ = weak incomplete membranous staining in <10% of the tumour cells; 2+ = incomplete moderate intensity membranous staining in ≥10% of the tumour cells; and 3+ = uniform intense complete membranous staining. Immunohistochemistry showed negative staining for all three surrogate markers and confirmed triple negative nature of the tumour developed in the treated rats. Figure 4 shows negative staining for ER (Figure 4a), PR (Figure 4b) and HER2+ (Figure 4c) anti-bodies.

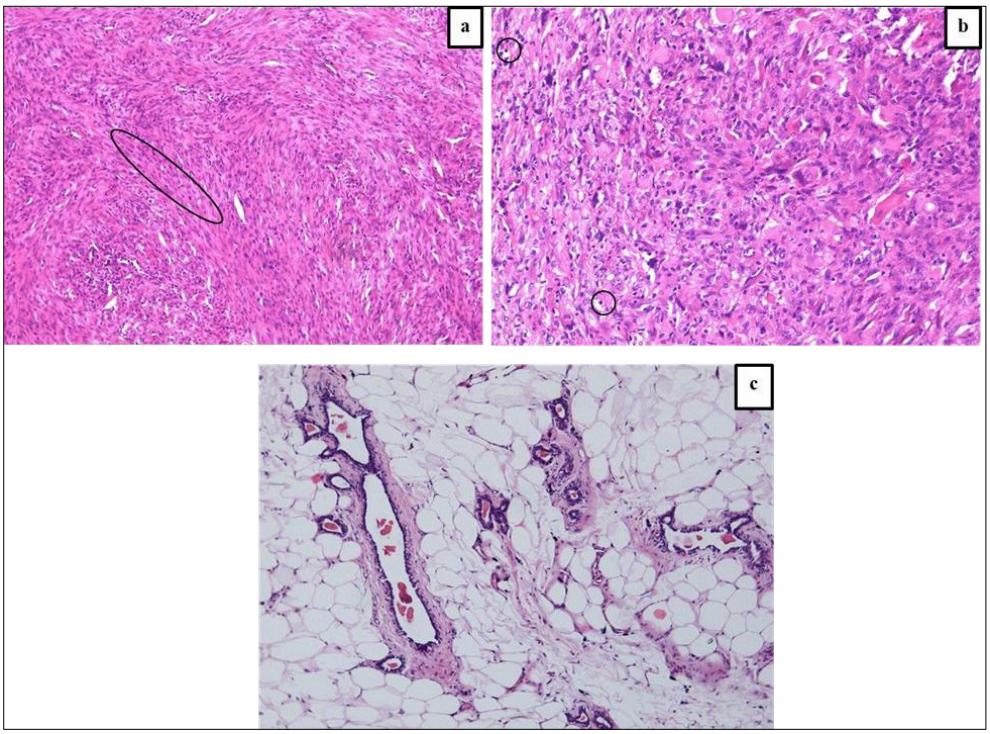

Figure 3 Histopathological observations of breast sections of control and experimental group (DMBA) of rats (Haematoxylin and Eosin staining) (a&b) shows H&E (10X) stained section of tumour in DMBA treatment group. Lymphocytes are marked in round shape, spindle shaped cells are marked in oval shape (c) shows H&E (40X) stained section of breast tissue in Control group

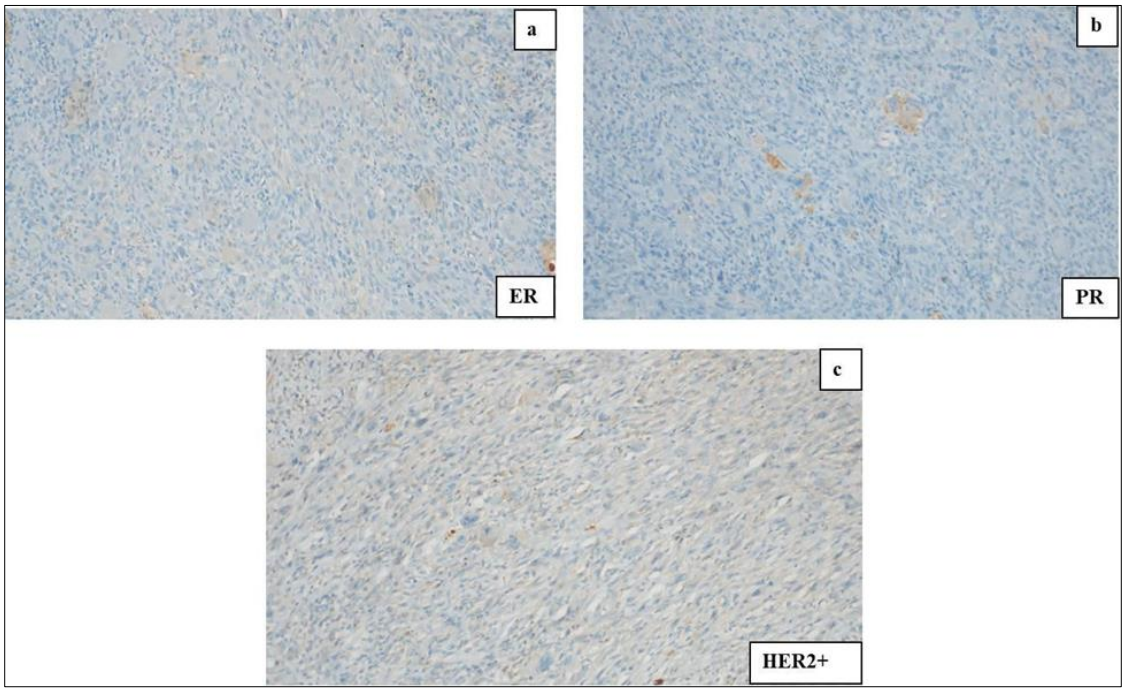

Figure 4 Immunohistochemical staining of breast sections of experimental group (DMBA treated rats). (a) ER staining (b) PR staining (c) HER 2+ staining

4. Discussion

The rat mammary gland is widely used to model mammary carcinogenesis as it resembles human breast in pathology and hormone dependence. The high incidence rate of tumour induction in different rat strains makes it an appropriate choice to study mammary carcinogenesis [15]. In the present study 7,12-dimethylbenz[a]anthracene (DMBA) a chemical carcinogen was used for the development of mammary tumours in rats (Female SD /Wistar) rats [13,14,16]. This model mimic's breast cancer pathology closely as the active metabolites have the capacity for damaging DNA molecules which is a main initiating event in mammary cancer progression [17-20]. DMBA is highly carcinogenic and lipophilic but requires metabolic activation for its carcinogenicity. It is converted into its active metabolites e.g epoxides which have a capacity for DNA damage thus initiating mammary carcinogenesis. Further, these compounds disrupt DNA repair through depurination, thereby inducing cell death pathways and tumour development in tissues [7]. Higher metabolic activity and epoxide formation is seen in types 1 and 2 lobules because of their higher cellular proliferative index [7, 21-23]. Several tissues including mammary gland are capable of activating DMBA therefore making it a susceptible site for cancer initiation. This susceptibility to DMBA is also strongly age-dependent as suggested by Alfredo et al. [24], maximum when the chemical is injected between the ages of 45-60 days, which is the age of the beginning of sexual maturity [25]. The chance of inducing breast cancer chemically is greater in this phase of the animal's life as there occurs active breast organogenesis and high rate of proliferation of type 1 and 2 lobules during this period. Therefore, in the current experiment DMBA was given to SD and Wistar rats at the age of 47 days either by gavage for DMBA or intraperitoneal injection. Tumours were induced with almost 100% incidence within 14 weeks in wistar rats. Among the rat strains used for the study (SD and Wistar) Wistar-Furth rats were found to be more susceptible to DMBA insult. However, the genetic basis for different susceptibilities to mammary tumour induction in different rat strains are still unclear [15, 26].

Further various oxidative stress parameters and histopthological studies were performed to confirm the development of mammary carcinoma. DMBA toxicity was also assessed in various organs including breast, liver lungs and kidney using biochemical assays. As per results a significant decrease in GSH levels and elevated MDA, ROS levels were observed in tissue collected from different organs of DMBA treated rats (Liver, kidney, breast and lungs). DMBA-induced liver damage is closely associated with the increase in LPO and GSH depletion through a generation of ROS and alterations in the activity of antioxidant enzymes. Results also indicated a significant increase in Liver marker enzymes SGOT and SGPT indicating hepatotoxicity. Our results are in line with the results obtained by Batcioglu et al [13] and Mani et al [14]. The H&E staining of rat mammary tissue showed development of metaplastic triple negative breast cancer after continuous injection or dose of drugs for time. Immunohistopathology studies confirmed ~~showed~~ triple negative nature of mammary tumour developed in the rats. The results are in line with histopathological examination reports in mammary cancer by Liska et al [4].

5. Conclusion

Considering the different complex pathways involved in breast cancer development and progression different kind of models will be needed to understand the underlying molecular pathway. But this experimental animal model closely mimics human breast cancer and can be used as a comparative group in further studies with the purpose of elucidating the role of biomodulation in mammary carcinogenesis.

Compliance with ethical standards

Acknowledgments

The authors gratefully acknowledge the staff of the Central animal house and department of Biophysics, PGIMER, and Chandigarh.

NS and AN: performed the experiments, recorded and analysed the data, DK and AB: performed the histopathological study, scoring and data analysis SB: designed and supervised the study. All authors participated in writing and editing the manuscript.

The study was conducted with department resources and no extramural funding was received from any funding agency.

Disclosure of conflict of interest

The authors do not have any competing interests in the manuscript.

Statement of ethical approval

All experiments were done with written approval from the Institute animal ethics committee and all guidelines for maintenance and use of animals were followed.

References

[1] Harbeck N, Penault-Llorca F, Cortes J et al. Breast cancer. Nat Rev Dis Primers. 2019; 5: 66.

[2] Sakamoto K, Schmidt JW, Wagner KU. Mouse Models of Breast Cancer (2018) Methods Mol Biol. 2015 ; 1267: 47–71.

[3] Kirubha SPA, Anburajan M, Venkataraman, Akila R, Sharath D, Baldev Raj. Evaluation of Mammary Cancer in 7, 12-Dimethylbenz(a) anthracene-Induced Wister Rats by Asymmetrical Temperature Distribution Analysis Using Thermography: A Comparison with Serum CEA Levels and Histopathology. J Biomed Biotechnol 2012; 11.

[4] Líška J, Brtko J, Dubovický M, et al. Relationship between histology, development and tumourigenesis of mammary gland in female rat. Exp Anim. 2016; 65(1): 1-9.

[5] Akbari Bazm M, Naseri L, Khazaei M. Methods of inducing breast cancer in animal models: a systematic review. WCR.J. 2018; 5(4): e1182

[6] Saminathan M, Rai RB, Dhama K, Jangir BL, Suresh S. Effect of Morinda citrifolia (Noni) fruit juice on antioxidant, hematological and biochemical parameters in N-methyl-N-nitrosourea (NMU) induced mammary carcinogenesis in sprague-dawley rats. Int J Pharmaco.l 2014; 10: 109-119.

[7] Gao J, Mitchell LA, Lauer FT, Burchiel SW. p53 and ATM/ATR regulate 7, 12- dimethylbenz [a] anthraceneinduced immunosuppression. Mol Pharmacol. 2008; 73: 137- 146.

[8] Petrelli F, Coinu A, Cabiddu M, Ghilardi M, Lonati V, Barni S. Five or more years of adjuvant endocrine therapy in breast cancer: a meta-analysis of published randomised trials. Breast Cancer Res Treat. 2013; 140(2): 233-40.

[9] Lowry OH, Rosebrough NJ, Farr AL. Protein measurement with the Follin's phenol reagent. J Biol Che. 1951; 193: 265-275.

[10] Ellman GL . Tissue sulphydryl groups. Arch Biochem Biophy. 1959; 82: 70-77.

[11] Wills ED. Mechanism of lipid peroxide formation in animal tissue. Biochem J. 1966; 99: 667.

[12] Best TM. Free radical activity, antioxidant enzyme, and glutathione changes with muscle stretch injury in rabbits. J Appl Physiol. 1999; 87: 74–82.

[13] Batcioglu K, Uyumlu AB, Satilmis B, et al. Oxidative stress in the in vivo DMBA rat model of breast cancer: suppression by a voltage-gated sodium channel inhibitor (RS100642). Basic Clin Pharmacol Toxicol. 2012; 111(2): 137-141.

[14] Mani G, Arumugam M, Mari1 A, Devaki1 T. Naringin Attenuates DMBA-Induced Mammary Carcinogenesis in Rats via Regulating the Oxidative Stress and Antioxidants Status Journal of Chemical and Pharmaceutical Research. 2018; 10(7): 44-54

[15] Cheung SY, Yuen MT, Choi HL, et al. An expression study of hormone receptors in spontaneously developed, carcinogen-induced and hormone-induced mammary tumours in female Noble rats. Int J Oncol. 2003; 22(6): 1383-1395.

[16] Huggins C, Grand LC Brillantes FP: Mammary cancer induced by a single feeding of polynuclear hydrocarbons, and its suppression. Nature 1961; 189: 204-207.

[17] Angeline Kirubha SP, Anburajan M, Venkataraman B, Akila R, Sharath D, Raj B. Evaluation of mammary cancer in 7,12-dimethylbenz(a)anthracene-induced Wister rats by asymmetrical temperature distribution analysis usingthermography: a comparison with serum CEA levels and histopathology. J Biomed Biotechnol. 2012; 786417.

[18] Dias MF, Sousa E, Cabrita S, Patrício J, Oliveira CF. Chemoprevention of DMBA- induced mammary tumours in rats by a combined regimen of alpha-tocopherol, selenium, and ascorbic acid. Breast J. 2000; (1): 14–19.

[19] Rajkumar L, Balasubramanian K, Arunakaran J, Govindarajulu P, Srinivasan N. Influence of estradiol on mammary tumour collagen solubility in DMBA-induced rat mammary tumours. Cell Biol Int. 2006; 30(2):164–168.

[20] Kaur J, Sharma M, Sharma P, Bansal M. Antitumour activity of lantadenes in DMBA/TPA induced skin Tumours in mice: expression of transcription factors. AJBS. 2010; 2(1): 79–90.

[21] Clarke R. Issues in experimental design and endpoint analysis in the study of experimental cytotoxic agents in vivo in breast cancer and other models. Breast Cancer Res Treat. 1997; 46: 255-78.

[22] Russo J, Larref MH, Balogh G, et al. Estrogen and its metabolites are carcinogenic agents in human breast epithelial cells. J Steroid Biochem Mol Biol. 2003; 37: 1-25.

[23] Balogh GA, Russo IH, Russo J. Mutations in mismatch repairs genes are involved in te neoplastic transformation of human breast epithelial cells. Int J Oncol. 2003; 23: 411-9.

[24] Barros Alfredo Carlos SD, et al . Induction of experimental mammary carcinogenesis in rats with 7,12-dimethylbenz(a)anthracene. Rev. Hosp. Clin. 2004 ; 59(5): 257-261.

[25] Grubbs CJ, Juliana MM, Hill DL, Whitaker LM. Suppression by pregnancy of chemically induced preneoplastic cells of the rat mammary gland. Anticancer Res. 1986; 6: 2395-401.

[26] Russo J, Gusterson BA, Rogers AE, et al. Biology of disease: comparative study of human and rat mammary tumourigenesis. Lab Invest. 1990; 62: 244-77.