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Full Length Research Article

Tumorigenesis in Immunosuppressed Sprague-Dawley Rats: A Case Study of Exposures to NMU Carcinogen and MCF-7 Cancer Cell Lines

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ABSTRACT
Breast cancer is a leading cause of morbidity and mortality worldwide. Reliable *in vivo* models are essential for studying tumor pathogenesis and therapeutic interventions. This study evaluated breast tumor induction in Sprague Dawley (SD) rats using either MCF-7 cancer cell lines or N-methyl-N-nitrosourea (NMU). Female SD rats were divided into three groups: one received 50 mg/kg NMU, another was injected with 0.1 mL containing 10^5 MCF-7 cells, and a third served as untreated control. All groups were immunosuppressed with ketoconazole and cyclosporine. Body weight and breast tissue palpation were monitored for 8 weeks. Hematological indices were assessed before and after immunosuppression. Finally, the rats were humanely sacrificed, harvested organs and breast tissues were examined histologically. Immunosuppression caused a significant reduction in neutrophils and lymphocytes ($p < 0.05$). Progressive weight loss was observed in the NMU and MCF-7 treated groups. Palpable breast tumors appeared after 6 weeks in NMU-exposed rats, with histology confirming malignant lesions and comedo necrosis. NMU also induced hemorrhages in the lungs and spleen, and hyperchromasia in cardiac and renal tissues, indicating systemic toxicity. No tumors were detected in MCF-7 treated rats within the same period, and organ damage was minimal. In conclusion, NMU at 50 mg/kg successfully induced breast tumors in SD rats within 6 weeks but also caused notable toxicity in non-target organs. These findings highlight the need for strategies to minimize systemic damage when developing *in vivo* breast cancer models.
KEYWORDS: Breast cancer; Immunosuppression; N-methyl-N-nitrosourea; Sprague Dawley rats; *In vivo* model

INTRODUCTION

Cancer is a collection of diseases characterized by uncontrolled, continuous growth and spread of abnormal cells throughout the body via metastasis. DNA anomalies are normally detected for correction and elimination by several cellular proofreading mechanisms. However, when abnormal DNA replication persists, the formation of malignant tumor cells drives cancer development (Leutholtz and Ripoll, 2011) and, as a consequence, normal cells are destroyed (Greenwell and Rahman, 2015; Siegel *et al*., 2016). Cancer is a leading cause of mortality worldwide as reported in the GLOBOCAN database, which indicated a rise in the global cancer incidence from 19.3 million cases in 2020 to nearly 20 million in 2022, with lung cancer remaining the most prevalent and the leading cause of cancer deaths worldwide (Cao *et al*., 2024). In women, breast cancer remains a major cause of malignancy-related deaths. In 2022 alone, there were an estimated 2.3 million women diagnosed with breast cancer and 670,000 deaths globally. Among the various types of cancer, breast cancer has emerged as the most frequently diagnosed malignancy globally. It accounts for 11.7% of all cancer cases and remains the leading cause of cancer-related deaths among women worldwide. In 2020 alone, approximately 2.3 million women were diagnosed with breast cancer, with 685,000 deaths recorded globally (Arnold *et al.,* 2022). The incidence rates vary significantly across regions, with rates ranging from 27.9 per 100,000 in Central Africa to 91.6 per 100,000 in Western Europe, reflecting differences in genetic factors, lifestyle patterns, and screening practices (Ferlay *et al*., 2021, Sung *et al*., 2021).

The burden of breast cancer in Africa presents a particularly concerning scenario. The continent faces unique challenges in cancer management, including limited healthcare resources, late-stage diagnosis, and inadequate treatment facilities. In sub-Saharan Africa, breast cancer incidence has been rising steadily, with approximately 129,000 new cases diagnosed annually. The mortality rate in this region is disproportionately high, with about 64% of diagnosed patients succumbing to the disease, compared to approximately 19% in North America (Adeloye *et al.,* 2018; Anyigba *et* al., 2021). Contributing factors include limited access to screening programs, cultural barriers, lack of awareness, and inadequate healthcare infrastructure. Nigeria, specifically, bears a significant portion of Africa's breast cancer burden. Current statistics indicate that breast cancer accounts for 22.7% of new cancer cases in Nigeria, with approximately 28,000 new cases diagnosed annually. The mortality rate is particularly alarming, with about 70% of Nigerian women presenting with advanced-stage disease at initial diagnosis (Jedy-Agba *et al.,* 2012).

The terminal ductal lobular unit (TDLU) of the breast is a functional unit that is believed to contain cell populations susceptible to chemical carcinogens from where breast

cancer typically starts to develop. Chemotherapy of breast cancer involves the combination of carefully selected cytotoxic agents, each targeting a specific phase of the cancer cell cycle. Thus, targeting breast cancer cells with cytotoxic agents proves to be challenging considering their co-existence with normal cells of the body. Such treatments, unfortunately complicate further, the burden of cytotoxicity patients have to endure. Recent technological advancements in cancer research, such as *in vitro* and *in silico* research methods, have achieved significant progress in understanding aspects of cancer metastatic mechanisms. These methods often study specific cancer cell types in isolation and under controlled environments. Replication of the pharmacokinetic and pharmacodynamic parameters as they apply in the natural environment of cancer cells and resistance to cell death, as supported by intrinsic mechanisms that promote tumor proliferation, angiogenesis, migration and invasion, and metastasis, remains a challenge (Li *et al*., 2021). This and other proliferation mechanisms of the disease further complicate the treatment approach.

Models that allow for comprehensive drug interaction with cancer cells in their natural environment not only provide information on drug-cancer cell cytotoxicity but also provide information on the unintended secondary adverse effects that may arise from the drug or its metabolites. Animal models for cancer study remain a viable option for pharmacological screening of cytotoxic agents. Specifically, breast cancer models developed from rodents have been used to screen both natural and synthetic compounds for their cytotoxic properties. A major difficulty, however, is the extremely short survivability period of these models, making them difficult to maintain for long-term studies or transfer over long distances between research labs. Therefore, development of *in vivo* models locally for cancer studies not only promotes continuous screening of potential anti-cancer agents but also affords low-budget research labs to follow through on progress made, especially in the area of anti cancer natural products identified via *in vitro* screenings. This paper is focused on exploring the use of N-methyl-N-nitrosourea (NMU) and MCF-7 cancer cell lines to induce breast cancer in immunosuppressed Sprague Dawley (SD) rats and assess signs of toxicity in non target tissues.

MATERIALS AND METHODS

All chemicals and reagents used for this study were analytical grade. NMU (CAS No. 684-93-5; Batch No. N82509963) was obtained commercially from Nanjing Forever Pharmacy Co., Ltd, Jiangsu Province, China, while MCF-7 cancer cell lines and SD rats were supplied by the Center for Advanced Medical Research and Training, Usmanu Danfodiyo University, Sokoto (UDUS).

Experimental Animals and Grouping

All experimental procedures were reviewed and approved by the Animal Ethics Committee of UDUS, and strict adherence to the recommended guidelines for the welfare and use of animals in the breast cancer model was adopted throughout the study. Apparently healthy female SD rats weighing 130 g to 200 g were acclimatized for one week before commencement of the experiment. The rats were randomly divided into three groups (n = 5), namely group I NMU NMU-bearing rat; group II MCF-7 bearing rat, and group III represents the normal healthy control.

Preparation and Administration of NMU and MCF-7 Cancer Cell Lines

NMU solution was prepared by dissolving 250 mg of NMU in 8 ml of warm normal saline (prepared by dissolving 8 g NaCl in 100 ml distilled water). The pH of the solution was adjusted to 4.0 using a few drops of 3% acetic acid, and the final volume was topped up to 10 ml with warm normal saline. The prepared NMU solution was wrapped in aluminum foil and maintained at 35 – 40 °C prior to administration. MCF-7 Cell line was incubated under standard conditions of 37 °C and 5% CO_2. The cell growth medium was changed regularly until 70 – 80 % cell confluence for sub-culturing was achieved. The cells were sub-cultured using 25 cm^2 culture flasks to maintain the cells and obtain several cell culture passages for *in vivo* induction. Cell counting was performed using a hemocytometer to determine the number of cells required for inoculation.

Immuno-Suppression of SD Rats

The SD rats were subjected to immune suppression prior to the induction of breast cancer. The rats were administered 10 mg/kg ketoconazole and cyclosporine orally for 7 days, after which 60 mg/kg cyclophosphamide was administered subcutaneously for 3 days. Blood samples were collected for haematological assessment.

Induction of Breast Cancer

Group I SD rats were administered NMU following the methods described by Thompson *et al.* (1992) and Rivera *et al.* (1994) with some modifications. Briefly, the freshly prepared 50 mg/kg body weight NMU solution at pH 4.0 was injected intraperitoneally (ip) after every three weeks. Palpation of the mammary gland was done twice per week for tumor detection. Experimental control female SDR received an equivalent volume of 0.8 % physiologic saline. Group II SD rats were inoculated with MCF-7 cell lines according to the methods of Pulaski and Ostrand-Rosenberg (2000) and Xanthopoulos *et al.* (2005). Exactly 0.1 ml equivalent of 10^5 cells suspended in PBS was injected subcutaneously in the right flank of the mammary fat pad of the immunosuppressed experimental animals. Booster shots were given every two weeks for the first month post inoculation, after which the mammary gland

was monitored for signs of tumor for 8 - 10 weeks period. The rats were humanely sacrificed after 10 weeks, and high blood-perfused organs – heart, lung, kidney, liver, and spleen - as well as the breast tissue were harvested and preserved in 10% buffered formalin for histopathological examinations.

Bodyweight of Experimental Rats

The bodyweight of the SD rats was monitored for a period of 8 weeks at a weekly interval.

Haematological Assessment of Immunosuppressed Rats

The total WBC, lymphocytes, and neutrophils of experimental rats were determined using an automated hematology analyzer XE-5000 at the veterinary clinical pathology lab of the Veterinary Teaching Hospital, Usmanu Danfodiyo University, Sokoto.

Tumor Volume and Histopathological Examination of Breast Tissues and Selected Organs

Tumor volume (V) per animal was measured using a vernier calliper. The tumor length and width were measured in millimetres and the tumour volume was calculated using the formula $V = ab^2/2$, where 'a' and 'b' represent the length and width tumour diameters, respectively (Dubben *et al*., 1998). Excised breast tissue and organs of interest of the SD rats preserved in buffered 10% formalin were processed using an automatic tissue processing machine (Leica TP 1020). The prepared 5-micron thickness sections of the tissues were then stained with hematoxylin and eosin (H & E) and viewed under a microscope at x100 magnification. A camera mounted on the microscope was used to capture photomicrograph images of the prepared slides.

RESULTS

Figure 1 presents the bodyweight of SD rats exposed to NMU or MCF-7 cell lines. A progressive drop is observed in exposed Groups I (from 210 g to 140 g) and II (from 182 g to 137 g, respectively) compared with the steady bodyweight increase in the control Group III.

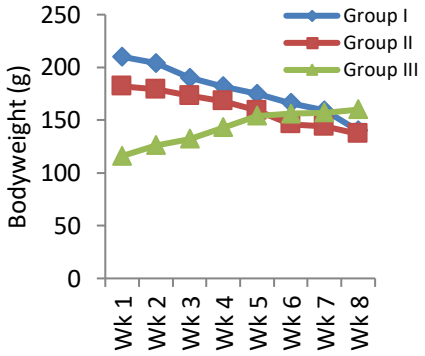

Figure 1: Weekly bodyweight of SD rats exposed to NMU

The haematological analysis of the SD rats' results is presented in Table 1. Exposures of the experimental rats to ketoconazole and cyclosporine significantly ($P < 0.05$) suppressed the immunity of the SD rats. There was a significant drop in WBC across the experimental groups.

Table 1: Haematological indices of immunosuppressed SD Rats

	NEUTROPHILS %		LYMPHOCYTES %		WBC x 10^3 (mm^3)	
Group	**Before**	**After**	**Before**	**After**	**Before**	**After**
Group I	26.00±1.73[a]	17.00±5.29[b]	80.67±1.00[c]	40.67±4.93[d]	22.20±6.10[e]	3.80±0.33[f]
Group II	27.67±2.52[a]	15.67±4.04[b]	79.00±3.00[c]	39.674.73[d]	19.40±2.10[e]	3.60±0.10[f]
Group III	25.00±7.00[a]	16.00±4.36[b]	81.00±6.08[c]	41.00±3.61[d]	21.00±4.51[e]	4.25±0.70[f]

The "before" and "after" examinations were done at a week interval of immunosuppression. Values with different superscript for each parameter pair-wise are significantly different at $P < 0.05$

Plate 1 shows palpable tumor (A) detected at 7 weeks post NMU exposure with an estimated tumour volume of 7.2 ± 0.23 cm^3 for SD rats. No palpable tumour nodules were observed in the MCF-7 inoculated SD rats after 8 weeks of monitoring. Image labeled C shows a micrograph of mammary tissue of healthy SD rats sections characterized by bi-layered ducts of luminal epithelial and basal myo-epithelial cells, adipose and fibrous fatty tissues. The image labeled D shows typical malignant lesions consisting of sheets of epithelial clusters with possible stromal desmoplasia (red arrows) and comido necrosis (yellow arrow).

Plate 2 presents thin sections of highly perfused organs, including the heart, kidney, liver, lung, and spleen of the experimental SD rats stained with H & E and viewed at x 100 magnification. Heart tissues of the healthy control and MCF-7 exposed SD rats revealed normal cardiocytes with normal staining nucleus (Black arrows respectively). Mild hyperchromasia of the cardiocytes nucleus (Blue arrow) but normal lumen of the vein (Black arrow) is seen in the NMU-exposed SD rats. Thin sections of the kidney for the healthy control suggest normal Bowman's capsule and normal capsular space (black arrows) as well as normal proximal convoluted tubule (blue arrow).

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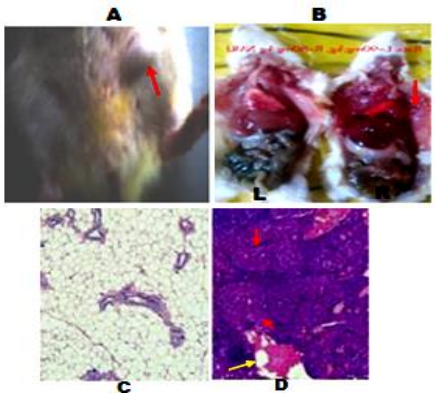

Plate 1: Histomorphological and microtome sections of the breast tissues.
Image (A) shows a palpable marginal tumour nodule in NMU-exposed SD rats. Image (B) is the autopsy of healthy (labeled L) and NMU-treated rats (Labeled R with red arrow showing tumour nodules on the right). Images (C) and (D) represent thin sections of breast tissues of healthy control and NMU-exposed SD rats, respectively. Images are H & E-stained and viewed at x 100 magnification.

The NMU-exposed group showed signs of hemorrhage (blue arrow), gromeruli hyperchromasia, narrowed capsular space (black arrow) but normal proximal convoluted tubules. Gromeruli infiltration of inflammatory cells is observed in the kidney section of the MCF 7 exposed group, congested Bowman's capsule (black arrow) and a normal proximal convoluted tubule (blue arrow).

Histopathological examinations of the liver revealed that the healthy control group had hepatocytes with normal hepatic sinusoids (blue arrow) and a patent central vein (black arrow), as well as a normal portal triad (red arrow). Similar observations were made for the NMU-exposed and MCF-7 SD rats, which showed normal hepatocytes with normal hepatic spaces (blue arrow), normal patent central vein (black arrow), and normal portal triad (red arrow). Observations on the lung tissue of the experimental rats show normal patent bronchioles (blue arrow) and alveolar spaces (black arrow). Localized necrotic tissues with mild haemorrhage around the interstitial spaces (green arrow) were observed in the lungs of the NMU-exposed, but with normal epithelial lining (blue arrow) and alveolar spaces (black arrow). Haemorrhages were also observed in the thin sections of the lungs of MCF-7 inoculated SD rats. Thin sections of the spleen for the normal healthy control and MCF-7 exposed rats were observed to have normal white (black arrow) and red (blue arrow) pulp cavity filled with normal granulocyte and arteriole, while necrotic tissues and haemorrhages (green arrow) were observed in the thin sections for NMU exposed rats.

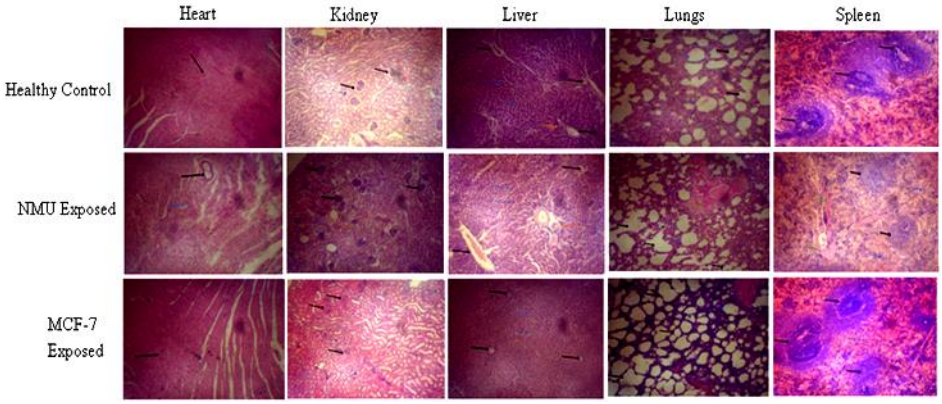

Plate 2: Thin sections of selected organs of SD rats exposed to NMU and MCF-7 Cancer cell lines. Tissues were subjected to H & E staining and viewed at x 100 magnification.

DISCUSSION

This study explores the use of cancer cell lines or chemical carcinogens (NMU) for developing *in vivo* cancer models. The SD rats in this study responded positively to ketoconazole and cyclosporine immune-suppression within 7 days of administration from a range of 19.40 ± 2.10 – 22.20±6.10 x 10 mm^3 to between (3.60±0.10 – 4.25±0.0 x 10 mm^3). Ketoconazole inhibition of cytochrome P450 3A prolongs the bioavailability and half-life of cyclosporine (Anderson and Blaschke, 1986), a potent immunosuppressive drug that inhibits T-cell activation and proliferation (Liddicoat and Lavelle, 2019). Despite achieving immunosuppression, the MCF-7-exposed SD rats did not develop palpable tumors after 6 weeks of observation, even though there was a progressive drop in body weight of the MCF-7-exposed rats, probably suggesting a delayed phenotypic expression of the carcinogenic process. This may not be unrelated to the less adaptable xenografting of MCF-7 cell lines in murine species or failure to initiate neoplastic transformation in a normal cell, leading to localized tumorigenesis. Moreover, xenografting is a time-dependent process usually lasting 90 to 120 days before nodules begin to appear. There is also the risk of susceptibility of immunosuppressed rats to

secondary infections, which may cause premature death of experimental animals. Studies have shown that orthotopic 4T1 mouse cell lines are more aggressive, hence attainable as cell implants for tumor induction in rats (Welsh, 2013; Herndon *et al*., 2024). Within the same time frame, however, palpable tumors developed 6 weeks post NMU injection. The nitrosourea compound induces mammary tumour through direct DNA alkylation point mutations, thereby activating oncogenes responsible for in situ tumorigenesis (Ariazi et al., 2005). Thin sections of the NMU-induced tumor revealed malignant lesions, stromal desmoplasia, and comido necrosis. Histological observations of non target organs, including the heart, kidney, lungs, liver, and spleen, suggest highly perfused organs may show slight to moderate injuries, as haemorrhages were noticed in thin sections of the lungs and spleen, and hyperchromasia in cardiocytes and renal tissue. Thus, the use of chemical carcinogens requires strict monitoring of laboratory processes towards minimizing side effects or systemic injuries that may compromise cancer model development or non cancer related mortality. Studies, however, suggest NMU has a high affinity to the mammary gland and coupled with the susceptibility of the breast tissues to DNA damage repair errors (Murray et al., 2009; Davis and Lin, 2011), there is an increased chance of low doses of NMU to induce carcinogenesis, provided the inoculation sites target established breast tissues.

NMU breast cancer model has the advantage of expressing genes similar to those in human breast cancer, closely mimicking anticipated molecular and biochemical responses (Chan et al., 2005) such as responsiveness to estrogen signaling (Nicotra *et al*., 2024), thus making the model suitable for pharmacological screening of anti-breast cancer agents. Establishment of in-house protocols for *in vivo* cancer models will not only be cost-effective but also allow researchers to conduct high-throughput screenings of natural or synthetic anticancer agents as well as translate *in vitro* findings into comparative *in vivo* pharmacological outcomes.

CONCLUSION

Administration of 50 mg/kg NMU (i.p.) caused phenotypic appearance of palpable tumors 6 weeks (p.i.) in immunosuppressed Sprague Dawley rats, accompanied by signs of toxicity to tissues that are highly perfused with blood, including the heart, kidney, liver, and lungs. MCF-7 inoculation requires a greater than 6-week period for a localized tumor in Sprague Dawley rats to appear. A high MCF-7 cell count coupled with sex steroid administration or utilization of more aggressive cell lines could be advantageous for breast cancer induction, especially when damage to non target organs is of major concern in the development of *in vivo* breast cancer models.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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