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Research Article

Pattern of N-Methyl-N-Nitrosourea (MNU) Induced Histotoxicity in Respiratory, Urinary and Reproductive Organs of Mice

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Abstract

Animal models are important in the study of cancer pathogenesis. N-methyl-N-nitrosourea (MNU) is a potent human carcinogen. Tumor induction by a single intraperitoneal (i.p.) injection of MNU was studied in Swiss albino mice to determine the histotoxic effects on selected organs of the respiratory, urinary and reproductive systems. Eighty mice, (40 males and 40 females) were randomly divided into four groups of 20 each. The test groups (twenty males and twenty females) were given i.p. injections of 50mg/kg of MNU, while the other two groups served as comparative control. They were monitored for seven months. One mouse from each of the 4 groups was sacrificed monthly over the exposure period. Selected organs (lungs, kidney and connective tissue around the mammary glands) were removed, fixed in 40% formal saline and processed using standard histological techniques. Mortality in the exposed animals was 1.25%. Organs of the exposed groups showed histopathological changes such as hyperplasia, infiltration of lymphocytes and collapsed alveolar sacs in the lungs; while vacuolation, vesicular and pleomorphic nucleation and increased nucleo-cytoplasmic ratio were observed in the kidneys. Lymphocyte infiltration was also observed in the glandular lumen of the connective tissues of the mammary gland, while organs of the control animals showed normal architecture. The degenerative changes observed are illustrative of the progression to cancer (carcinogenesis) that usually follows exposure to carcinogens in the environment. Interaction between the carcinogen and living cells resulted in damage to the organs of the exposed mice as evidenced by the distinct histopathological changes observed.

Keyword: *Carcinogenesis, N-methyl-N-nitrosourea, Histology, Swiss mice*

*INTRODUCTION

Animal models can help in the study and investigation of human carcinogenesis due to the similarities in cancer pathogenesis (Medina and Thompson, 2000). N-methyl-N-nitrosourea (MNU) is a highly potent carcinogen, mutagen, and teratogen (Macejova and Brtko, 2001). It is an alkylating agent which requires no metabolic activation and exhibits its toxicity by transferring its methyl group to nucleobases in nucleic acids. MNU models show reliability of tumour induction, organ site specificity, tumours of ductal origin and predominantly carcinomatous, histopatholological characterization, tumours of varying hormone responsiveness, and the potential to examine tumour initiation and promotion processes (Thompson and Adlaka, 1991).

Tumor induced cancer tissue has a distinctive appearance under the microscope such as a large number of dividing cells, variation in nuclear size and shape, loss of specialized cell features and normal tissue organization, and a poorly defined tumor boundary. Histological examination is a necessary diagnostic tool for carcinomas, as the definitive diagnosis of most malignancies must be confirmed by histological examination of the cancerous cells (Maton, 1993; Shuan-Li and Kuo-Ming, 2015). Several studies have been carried out on MNU by various researchers such as Grubbs *et. al.*, (1981), Shisa and Hiai (1985), Rivera *et al.*, (1994), Takahashi *et. al.*, (1995), Thompson *et.al*, (1995), Franchi *et al.*, (2003), Gal *et al*, (2006), Esendagli *et al*, (2008), Morton *et al.*, (2008) and Akanni *et al.*, (2010). However, these studies focused on Hamsters, Sprague-Dawley rats, Wistar rats and transgenic rats and mice such as F334/DUCRJ rats, BUF/N rats, Crj BDFI and P 53+/- mice. Limited information exists on the effects of MNU in Swiss albino mice. This study therefore provides information in this regard. This is because different strains may show differences in susceptibility due to genetic variation (Festing, 2010).

The aim of our study was therefore to determine the effects of MNU on the histopathology of selected organs and tissues of the respiratory (lungs), urinary (kidney) as well as the reproductive (connective tissues around the mammary gland) systems in the mice. This study complements earlier work by our group (Osowole *et al*, 2013) which provided information on the effects of MNU induction on the haematology as well as the histology of selected organs of the circulatory, lymphoid and digestive systems in Swiss albino mice.

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MATERIALS AND METHODS

Experimental design: A total of eighty (80) albino male and female mice, forty each, aged between seven to eight weeks old were purchased from the animal breeding house of the Department of Veterinary Physiology, Faculty of Veterinary Medicine, University of Ibadan, Ibadan. The experimental animals were grouped into two: Control Group (CG) and Exposed Group (EG), with each group comprising twenty male and twenty female mice respectively. The mice were quarantined in plastic cages in a pathogen free, well ventilated room under standard conditions of humidity, temperature and 12h light/dark cycle in the animal house of the Department of Zoology. They were acclimatized for two weeks prior to the commencement of the experiment. The mice were fed with mouse pellets obtained from Ladokun Feeds (Ibadan) and given water *ad libitum.* After acclimatization, the EG group were injected with a single intra-peritoneal dose of MNU (50 mg/kg), while the CG served as the comparative control. Thereafter, they were monitored for seven months. Weight changes of the mice in the EG and CG groups were recorded weekly and descriptive statistics i.e. Mean ± SEM and ranges were also determined. Mice were palpated thrice a week, throughout the duration of the exposure period to determine presence or otherwise of tumors. Four mice were sacrificed once a month from each group of male and female animals from both the CG and EG groups. The animals were anaesthetized with chloroform, and sacrificed by cervical dislocation, after which they were dissected and the organs harvested and stored in 40% formal saline. The experiment was terminated on the 28th week (i.e. seven months) after the induction of the mice. A standard protocol was drawn up in accordance with the Good Laboratory Practice (GLP) Regulations of the WHO (1998). The "principles of laboratory animal care" [NIH Publication No 85-23, 1985] were also followed in this study.

Test Substance: The carcinogen, N-methyl-N-nitrosourea (MNU Sigma Chemical Co., St. Louise Mo, Catalogue No. 684-93-5) was purchased from Sigma-Aldrich Japan, Tokyo. Approximately 0.04g of MNU was weighed in a sensitive weighing balance dissolved immediately prior to its use in 0.9% NaCl adjusted to pH 4 with acetic acid and stirred to ensure rapid dissolution. The calculated dosage of MNU, which was approximately 0.1 ml was then administered to the mice via intra-peritoneal injection.

Histological studies: Animals were sacrificed by anaesthetizing them with chloroform, after which they were dissected to remove the organs of interest. The kidney, the lungs and connective tissues around the mammary glands were removed and fixed in 40% formal saline for histological examination in order to preserve the tissue from degradation, and to maintain the structure of the cell and sub-cellular components. Subsequently, the samples were transferred to a cassette to allow the reagents to freely act on the tissue inside. This cassette was later immersed in multiple baths of 70%, 95% and 100% of alcohol respectively for the purposes of dehydration. This was followed by the addition of a clearing agent (xylene) to remove the alcohol. Samples were subsequently impregnated and finally embedded in molten paraffin wax using a Leica tissue embedding machine model EG 1160. The tissues were then allowed to solidify in the paraffin wax, so that thin sections of the tissues could be cut.

Several sections of 4 µm were trimmed and cut using a Leica rotary microtome model 2125 RT and mounted on thin transparent plastic slides coated with polylysine (Sigma Chemical Company, St. Louis, MO). Other equipment used for tissue processing for histological examination included a Raymond Lamb tissue floatation bath model E652, a hot plate model E18 and a Tissue-Tek II tissue processor model 4634. The sections were stained with haematoxylin, a basic dye which stains the nucleus blue, left for few minutes and counter stained with eosin, an acidic dye which stains the cytoplasm pink (H & E). Histological processing was carried out at the Department of Oral Pathology, College of Medicine, University of Ibadan, Ibadan. The prepared slides were viewed using an Olympus CX31 biological microscope fitted with an Olympus digital camera E-330 and provided with computer attachment at HATISS laboratories, Ososami, Oke-Ado, Ibadan.

Descriptive Statistics

Results of the body weight of the mice were expressed as Mean± Standard Error of Mean (SEM). Differences between the control and induced group of both sexes were determined separately using the students t-test and differences considered significant at the 95% level ($p<0.05$).

RESULTS

Table 1 shows the change in body weight in both male and female control and induced mice over the study period. Both groups gained weight over the study period. However, the male and female control mice showed a higher percentage weight gain than their induced counterparts (Table 1). Furthermore, the mean body weight of the control mice did not however differ significantly at $p<0.05$ from the respective induced group of both sexes, although the males generally showed a higher mean weight than the females (Table 1). Mortality rate recorded was 1.25%, as one male mouse from the induced group died after eight weeks of induction with the carcinogen. Except for a few induced mice which showed limited movement and decreased activity, majority of the induced mice appeared healthy and showed no observable differences from the control.

Histopathology

Lungs: Plate 1a shows the lungs of a female control mouse showing normal architecture of the alveolar sacs. Normal architecture of the respiratory bronchioles was also observed in the photomicrograph of the lungs of a control male as shown in Plate 1b. There were varying degrees of lesions observed in the histopathological examination of albino mice injected with the carcinogen. Hyperchromasia, lymphocyte infiltration and severe hyperplasia were the abnormalities observed in the lungs of an exposed female as shown in Plate 1c; while abnormal features observed in the lungs of a male induced mouse included bi-nucleated cells, collapsed alveolar sacs along with infiltration of inflammatory cells (Plate 1d).

Kidneys: Plate 2a shows the cortex of the normal kidney of a control male with the glomerulus filled with red blood cells, oval shaped nucleus with prominent nucleoli, Bowman's capsule and proximal convoluted tubules.

Table 1:
Change in body weight in control and induced albino mice over the study period

Experimental group	Initial weight before treatment (g)	Final weight after 28 weeks of treatment (g)	Percentage change in weight over the study period	Mean ± SEM
Male Group				
Control Male (CM)	20.43	40.37	+97.6%	34.23 ± 1.08
Induced Male (IM)	24.47	40.03	+63.6%	34.38 ± 0.94
Female Group				
Control Female (CF)	18.35	35.04	+91.0%	28.48 ± 0.95
Induced Female (IF)	19.03	31.61	+66.1%	27.24 ± 0.73

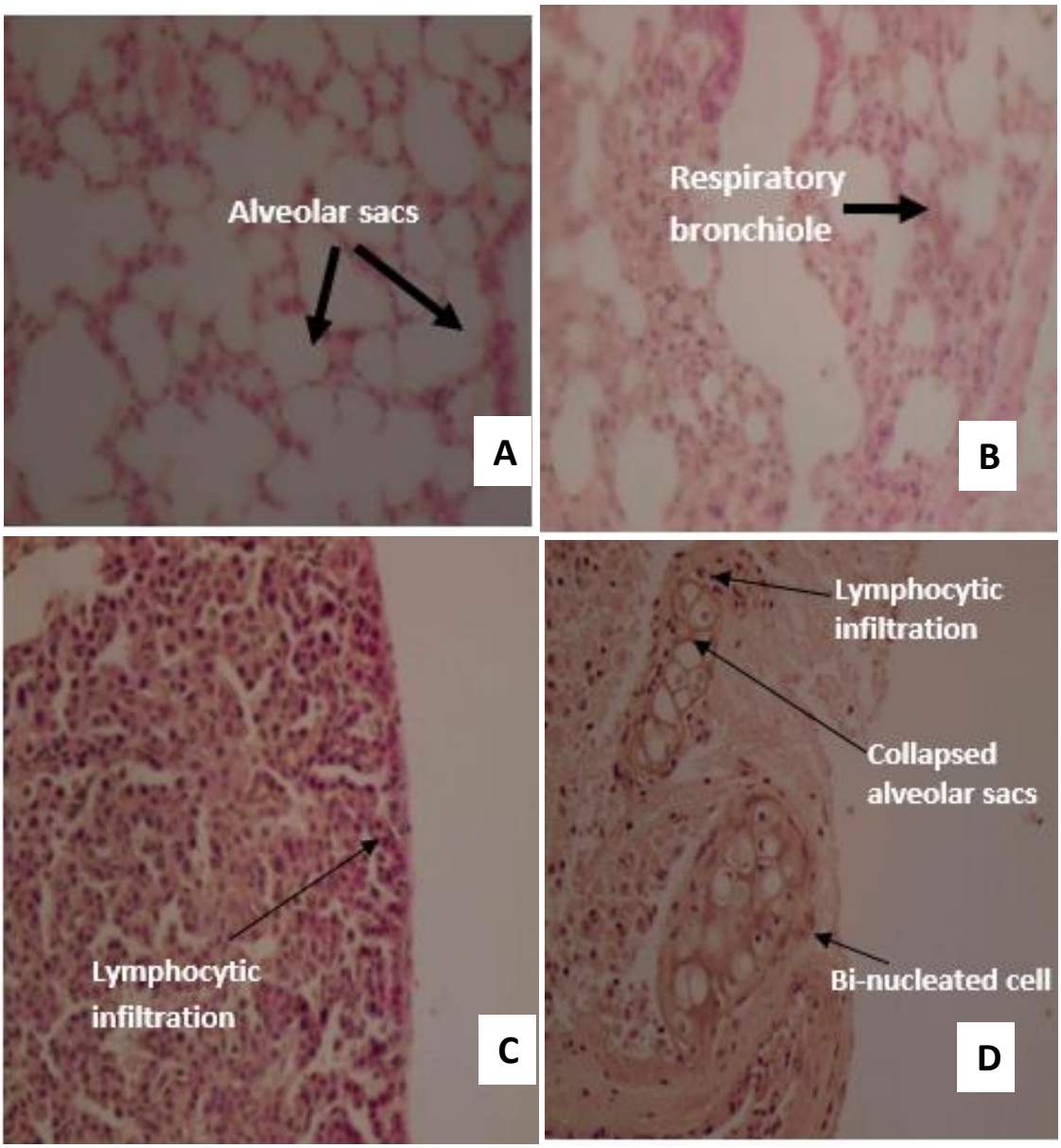

Plate 1
Photomicrograph of section of Lungs of (A)control female and (B)control male mice showing normal architecture H& E (X40). (C) : Photomicrograph of section of lung of an MNU-induced female mouse showing hyperchromasia (excessive pigmentation); lymphocytic infiltration and severe hyperplasia (excessive rate of cell division leading to unusually large number of cells); (D) Photomicrograph of section of lungs of an induced male mouse showing collapsed alveolar sacs, bi-nucleated cells and lymphocytic infiltration H & E (X40).

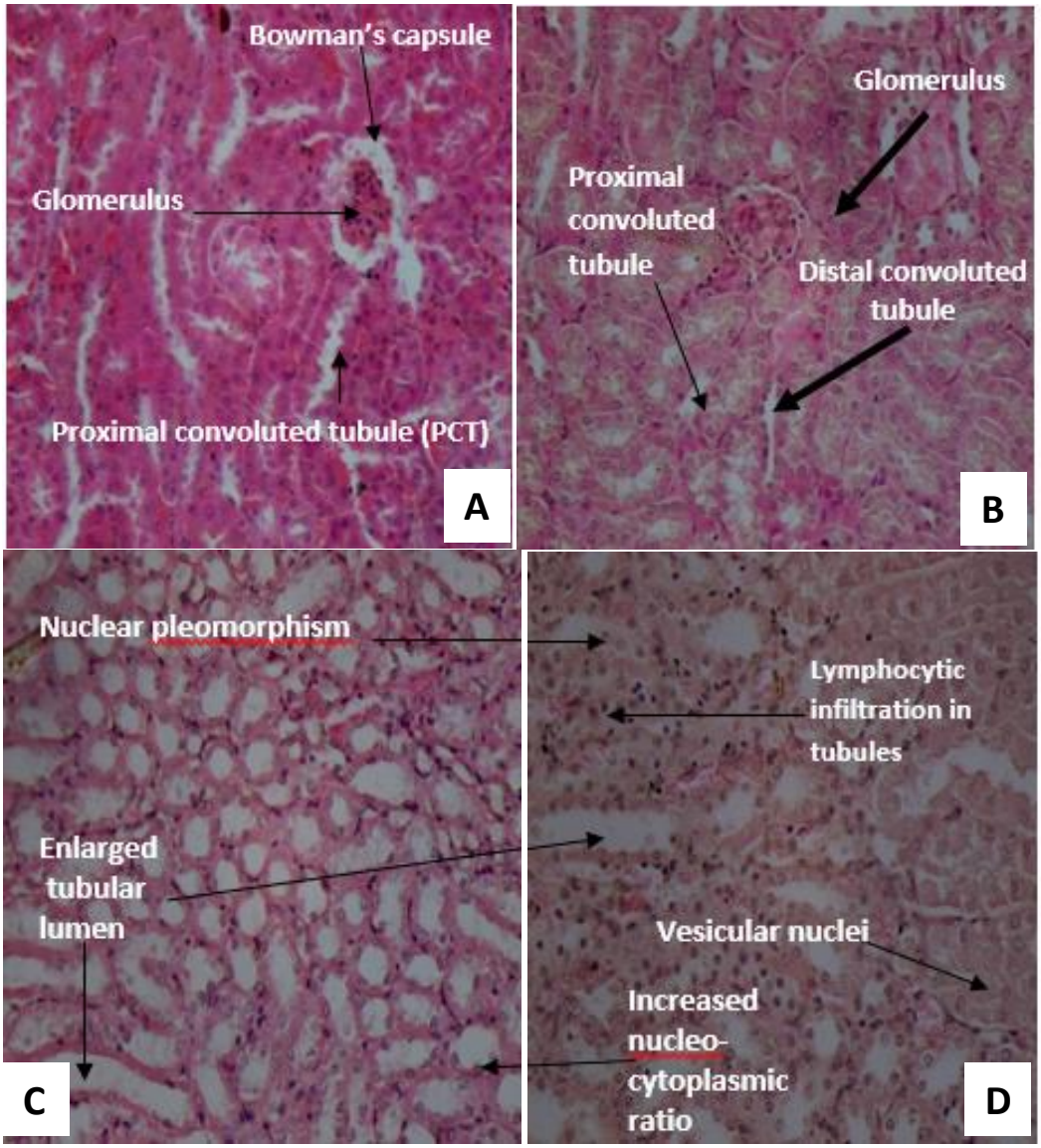

Plate 2
Photomicrograph of section of kidneys of (A)control female and (B)control male mice showing normal architecture H& E (X40). Photomicrograph of induced female (C) and tumor bearing male mice (D) showing enlarged tubular lumen, lymphocytic infiltration, vesicular nuclei, pleomorphic nuclei and increased nucleo-cytoplasmic ratio H & E (X40)..

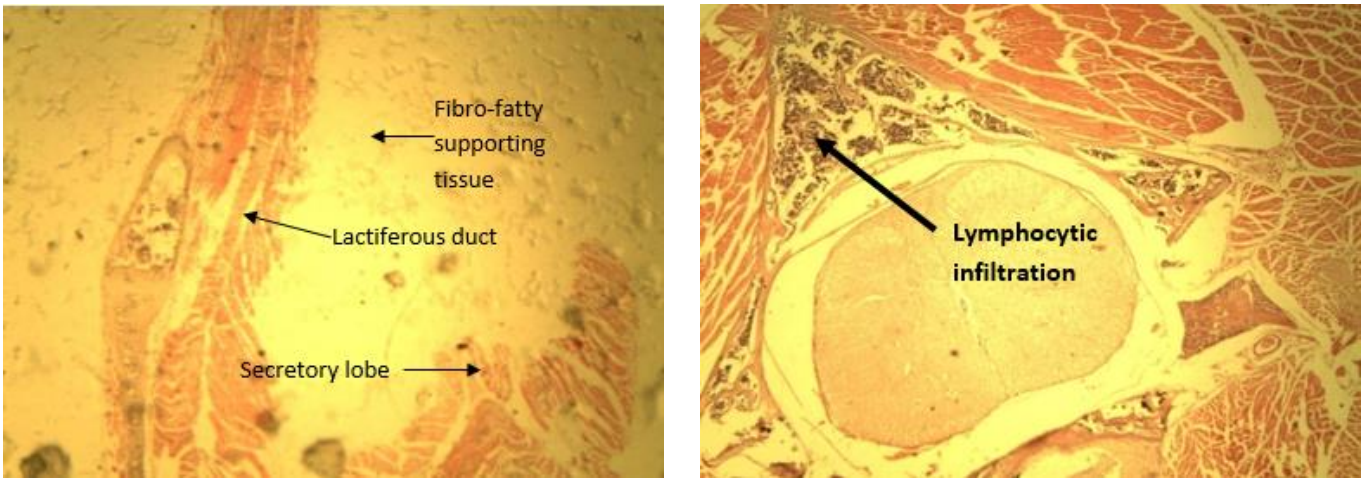

Plate 3:
Connective tissues around the mammary gland of (A) control female mouse showing normal architecture (B) induced female mouse showing glandular lumen filled with lymphocytes H & E (X40).

The kidney of a female control mouse also showed similar features as the male control with normal architecture (Plate 2b). The cortex of the left kidney of a female induced mouse showed enlarged tubular lumen (Plates 2c and 2d); while lymphocyte infiltration, vesicular and pleomorphic nuclei (varying sizes and shapes of the nucleus) and increased nucleo-cytoplasmic ratio were the abnormal features observed in the kidney of a male induced tumor bearing mouse (Plate 2b).

Connective tissues around the mammary glands: Connective tissues around the mammary gland in the control showed normal features such as the fibro-fatty supporting tissue, lactiferous duct and secretory lobes, while those of an induced female showed abnormal features such as infiltration of the glandular lumen with numerous lymphocytes and macrophages (Plates 3a and 3b).

DISCUSSION

This study showed varying degrees of histopathological alterations in animals exposed to the carcinogen, while the control animals showed normal histological features and confirmed as normal features as shown by Eroschenko, (2008). The observed abnormalities were probably due to the inability of the animals to metabolize and excrete the carcinogen. This in turn may have resulted in toxicant accumulation and a gradual onset of carcinogenesis in the MNU-induced animals. This is confirmed by the histopathological alterations and the development of a suspected tumor in the cervical region of an exposed male mouse two months after carcinogen induction. This eventually led to the death of the animal and the recorded percentage mortality of 1.25% (reported in Osowole *et al*, 2013). Histological examination is a major definitive and diagnostic tool for the confirmation of carcinomas. Other methods include immune-histochemical and molecular studies (Shuan-Li and Kuo-Ming, 2015).

The lungs is a very common site for metastasis from tumors in other parts of the body (Pandi *et al.*, 2016). Carcinogens may cause cumulative changes to the DNA in the bronchial epithelium of the lungs. As more tissues become damaged, a tumor may develop (Vaporciyan *et al.*, 2000; Dishop and Kuruvilla, 2008). In the present study, the lungs of the MNU induced mice showed histopathological alterations such as hyperchromasia, lymphocyte infiltration, hyperplasia, stroma formation infiltrated with bi-nucleated cells and collapsed alveoli sacs. Gal *et. al*, (2006) in their studies on mammary induced carcinogenesis by MNU in Wistar rats also observed interstitial hyperplasia in the lungs of exposed rats. Hyperplasia refers to increased cell proliferation and it is a common pre-neoplastic response to a stimulus (Zachary and McGann, 2013). Other abnormal features observed in that study included the invasion of polymorphic nuclei in the alveolar basement membrane which resulted in papillary alveolar protrusions; and acidophil or foamy cytoplasm in the carcinogen-exposed lungs (Gal *et al*, 2006). Although these additional abnormalities were not observed in the lungs of induced animals in this study, histopathological alterations such as hyperchromasia are definitive features of malignancy and is suggestive of abnormal mitotic divisions as indicated by Ki-Kwan *et al*, (2014).

Gawish *et. al.*, (2012) in their studies on the adverse effects of nicotine, a toxic substance implicated in lung cancer observed varying degrees of histopathological alterations in which the alveoli showed an extensive destruction of the walls resulting in the formation of enlarged, irregular air spaces, while the architecture of the lung was not preserved. Similarly, our study showed collapsed alveoli sacs in an induced male, possibly due to the adverse effects of the carcinogen. Lymphocytes are involved in the pathogenesis of various chronic inflammatory diseases. They are often recruited into tumors in a bid to control its growth (Jerome *et al*, 2008). Lymphocyte infiltration has been reported in carcinomas (Kawata *et al*, 1992) and was observed in the lungs of induced animals in this study.

The mammalian kidney is a target organ for a wide variety of toxic agents due to its prime function in the urinary system. It also serves homeostatic functions such as the regulation of electrolytes, maintenance of acid-base balance, and regulation of blood pressure (Cotran *et al.*, 2005). The kidney of MNU induced mice showed pathological abnormalities such as enlarged tubular lumen, lymphocyte infiltration, vesicular and pleomorphic nuclei, and increased nucleo-cytoplasmic ratio. Irregularly shaped or pleomorphic nuclei is a diagnostic feature of definitive malignancy (Ki-Kwan *et al*, 2014). Enlarged nuclei and nuclear pleomorphism are features of neoplastic cells as indicated by Ming-Juan *et al*, (2016). Furthermore, morphological distinct neoplasms in studies of Shuan-Li and Kuo-Ming, (2015) were characterized by cells with enlarged nuclei, distinctive nucleoli and eosinophilic cytoplasm. Thus, these findings may be indicative of a gradual onset of carcinogenesis as a result of exposure to the carcinogen - MNU and are possibly illustrative of the changes that occur in organisms following exposure to various environmental carcinogens.

Some authors have reported similar degenerative lesions in the kidney of rats and mice following exposure to various carcinogens (Mally *et al*, 2005; Akanji *et. al.*, (2008) and Morsy *et al*, (2012). Vacuolar degeneration, mononuclear cellular infiltrations and infiltration of interstitial cells in the nephrons were some of the abnormal features observed. In addition, Bespalov *et al*, (2001) observed the presence of mesenchymal tumors of the kidney when induced with MNU. However, this study did not show the presence of any tumor in the kidney, although a suspected tumor was observed in the cervical region of the dead animal (reported in Osowole *et al*, 2013).

This study also showed that the induction of MNU resulted in the infiltration of the glandular lumen with numerous lymphocytes and macrophages. Lymphocyte infiltration due to MNU induction is a possible indicator of the onset of carcinogenesis as lymphocytes invade the tissues in a bid to control tumor growth. Tumor infiltrating lymphocytes can be manipulated as a treatment for cancer (Jerome *et al*, 2008) and extensive lymphocyte infiltration has been associated with reduced tumor recurrence in some cancers such as hepatocellular carcinomas following resection (Kawata*et al*, 1992).

In conclusion, this study has shown various histopathological abnormalities such as multi-nucleation, increased nuclei sizes, nuclear pleomorphism, and lymphocyte infiltration amongst other alterations observed in the lungs, kidney and connective tissues around the mammary gland of Swiss albino mice injected intra-peritoneally with MNU, a potent carcinogen. These changes are possible indications of the gradual onset of carcinogenesis and are probably

illustrative of the degenerative changes that follow the exposure to various environmental carcinogens. The study also complements earlier studies by our group on the effects of MNU on the circulatory, lymphoid and digestive systems in Swiss albino mice, a strain less commonly used in animal carcinogenesis models. Interaction between the carcinogen and the living cells resulted to the degenerative changes observed in the organs of the induced mice.

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