Methylnitrosourea (MNU)—induced carcinogenesis and inflammation in some selected organs of female albino rats

Minari, J. B*. and Okeke, U.

Department of Cell Biology and Genetics, University of Lagos, Lagos, Nigeria

Abstract

Methylnitrosourea (MNU) is an alkylating agent which exhibits its toxicity by transferring its methyl group to nucleobases in nucleic acids, causing AT:GC transition mutations. It was originally designed as a chemotherapeutic alkylating compound, but later proven to exert direct carcinogenic, mutagenic and teratogenic potential. The carcinogenic effect of methylnitrosourea in some selected organs of female albino rats was evaluated using a modified protocol. Histopathological assessment of breast, liver, lungs and skin tissues of experimental animals was carried out using H and E staining procedure. Tumour markers, cancer antigen 15.3, 27.29 and carcinoembryonic antigen (CEA) in the blood of experimental animals were evaluated using an automated procedure. Histopathological examination revealed severe panniculitis in skin tissues, sinusoidal congestion in liver tissues, severe pulmonary inflammation in lung tissues, and stromal fibrosis in breast tissues. There was an increase in tumour marker levels in the blood of MNU induced rats compared to the controls group of rats. There was a significant difference between the values of CA 15.3 ($p < 0.01$) and CEA ($p < 0.05$) in rats induced with MNU when compared with the control. Cancer antigen 27.29 values showed no significant difference between the rats induced with MNU and control. Different forms of early stages of carcinogenesis were induced in female Albino rats using a novel and modified cancer induction protocol. Knowledge from this study did not only provide insight into possible harmful effects of MNU which could be obtained from foods containing nitrosamines, but it also provided the opportunity to test and prove a modified protocol of cancer induction which could be used to evaluate the preventive and therapeutic effect of different agents for human breast cancer within a short period of time.

*Author for Correspondence: baminjoe@yahoo.co.uk

Introduction

Methylnitrosourea (MNU), a pale yellow sand-like solid is an alkylating agent which exhibits its toxicity by transferring its methyl group to nucleobases in nucleic acids, causing AT:GC transition mutations (Klug et al., 2015). It was originally designed as a chemotherapeutic alkylating compound, but later proven to exert direct carcinogenic, mutagenic and teratogenic potential (Tsubura et al., 2011). NMU belongs to the group of compounds classified as nitrosamines (Sajjadi and Bathaie, 2016). Nitrosamines are found in foods such as smoked meat, soused meat, salami, sausage, fish meat,

cheese, and soy oil, in addition to cigarette smoke (Ashrafi et al., 2012). Nitrosamines are formed by the combination of nitrous oxide (NO) originating from nitrate (NO_3) and nitrite (NO_2) reacted with the secondary and tertiary amines formed by the destruction of proteins and amino acids in the gastrointestinal tract (Chan et al., 2005).

The carcinogenicity of methylnitrosourea (MNU) arises from its ability to methylate deoxyribonucleic acid (DNA) in aqueous environment (physiological pH) (NCBI, 2017). It donates its methyl group (CH_3) to the amino or keto groups in nucleotides altering base-

or keto groups in nucleotides altering base-pairing affinities which results to transition mutations (Klug et al., 2015). The molecular mechanism of NMU involves specific G-35 point mutation in codon 12 (HRAS gene) which results in substitution of normal glycine with an aspartic acid (Saminathan et al., 2014). A single dose of MNU has been shown to induce breast cancer in female Sprague Dawley rats (Yuri et al., 2003; Pula et al., 2013). Besides, MNU is also able to induce various cancers in experimental animals including retinal degeneration, esophageal, breast cancer, photoreceptor degeneration, gastric and colorectal malignancies (Leung et al., 2008; Takayama et al., 2008; Guru et al., 2013; Gonçalves et al., 2013; Chena et al., 2016; Sajjadi and Bathaie, 2016; Xiong et al., 2016). The carcinogenic potential MNU in some selected organs of female albino rats was evaluated in this study.

Materials and Methodology

Twenty female albino rats, 30 days of age were used in this experimental study. They were housed 10 animals per cage, were maintained under conditions of average 12 hours light: 12 hours dark. Experiment was carried out in the animal house of the Department of Cell Biology and Genetics, University of Lagos, Lagos, Nigeria in accordance with the rules in Nigeria governing the use of laboratory animals as acceptable internationally with ethical approval from College of Medicine, University of Lagos Health Research Ethics Committee (HREC).

Preparation of mnu

The carcinogen methylnitrosourea (MNU) was purchased by order from Hangzhou Sage Chemical Company Ltd, Hangzhou, China. It was dissolved shortly before administration in phosphate/citrate-buffered saline at pH 4.2 (1 part buffer to 14 parts saline).

Acute toxicity test

Acute toxicity was carried out following the protocol of Chinedu et al. (2013).

Induction of cancer in animals

Cancer was induced using a modified protocol of Sajjadi and Bathaie (2016). MNU was administered through intraperitoneal injection.

The experimental groups received 100mg/kg/body weight of MNU (four times) as stated below once per week for the first four weeks. Treatment of animals lasted for six weeks. The experimental and control animals were carefully checked daily and weight taken weekly. The rats were sacrificed at the end of the sixth week by cervical dislocation. Organs were harvested and fixed in formalin for histopathology. Blood was collected by orbital venous plexus bleeding in plain bottles kept in slanting position to induce separation of serum from whole blood and centrifuged (5000 rpm for 20minues) in plain sterile bottles for tumour markers evaluation (Parasuraman et al., 2010).

Group A: Rats that received 100mg/kg/wt of MNU for 6weeks

Group B: Rats that received distilled water (Control) for 6weeks

Statistical analysis

To analyse differences between the data obtained in the control group and the animals induced with MNU, the independent-sample test tool was applied using Statistical Product and Service Solutions (SPSS) version 23.0. All comparisons with P values below 0.05 were considered as significant.

Result

Table 1 shows the effect of methylnitrosourea (MNU) on the selected cancer specific antigen namely cancer antigen 15.3, (CA 15.3) cancer antigen 27.29 (CA 27.29) and carcinoembryonic antigen (CEA). There was a significant difference between the values of CA 15.3 ($p < 0.01$) and CEA ($p < 0.05$) in rats induced with MNU when compared with the control. Cancer antigen 27.29 values showed no significant difference between the rats induced with MNU and control.

The effects of MNU on the survival rate of experimental rat are shown Figure 3. No death was recorded in group A until the fifth and sixth week. No death was recorded in group B throughout the 6 weeks of the experiment.

The effect of MNU on the weight of experimental rats is shown in Table 2. There was an increase in weight in both groups but

there was no statistical difference between them ($p > 0.05$). However the rats in the control showed more increase in weight compared to the group induced with MNU.

Plate 1 shows the histologic section of skin tissue from rat induced with MNU (Plate 1A) and control (Plate 1B). Plate 1A shows infiltration of dermis and subcutaneous fat by dense aggregates of mixed inflammatory cell infiltrates which indicates a severe panniculitis. Plate 1B histologic section shows absence of inflammatory cell infiltrates within the underlying dermis and subcutaneous fat. No abnormalities are seen.

Histologic sections of liver tissue from rats induced with MNU (Plate 2A) and control (Plate 2B) is shown in Plate 2. Plate 2A shows radial plates of hepatocytes in which the hepatic sinusoids are packed with red cells showing sinusoidal congestion while Plate 2B shows hepatocytes arranged as radial plates with no fatty change, vascular congestion or infiltration of parenchyma by inflammatory cells.

Table 1: Effect of MNU on selected cancer specific antigen in experimental rats

CANCER ANTIGEN	GROUP A (µl)	GROUP B (µl)
CANCER ANTIGEN 15.3	5.33 ± 0.52	1.70 ± 0.20**
CANCER ANTIGEN 27.29	6.73 ± 2.00	3.73 ± 0.28
CARCINOEMBRYONIC ANTIGEN	0.90 ± 0.21	0.23 ± 0.13*

Values are means of 3 replicates $\pm$ S.E.M
Values carrying superscript (**) were significance ($p < 0.01$)
Values carrying superscript (*) were significant ($p < 0.05$)
KEY
Group A: Rats that received 100mg/kg/wt of MNU
Group B: Rats that received distilled water (Control)

Table 2: Effect of MNU on the average weight of experimental rats

S.N	GROUP A (g)	Weight change (g)	GROUP B (g)	Weight change (g)
1	44.15 ± 1.66		32.86 ± 0.66	
2	45.11 ± 1.97	22.59	55.45 ± 0.59	0.96
3	50.87 ± 2.27	-2.15	53.30 ± 0.69	5.76
4	52.88 ± 3.21	22.97	76.27 ± 0.93	2.01
5	55.06 ± 6.91	16.35	92.62 ± 1.49	2.18
6	55.35 ± 6.44	2.98	95.60 ± 1.87	0.29

Values are means of 10 replicates $\pm$ S.E.M
KEY
Group A: Rats that received 100mg/kg/wt of MNU
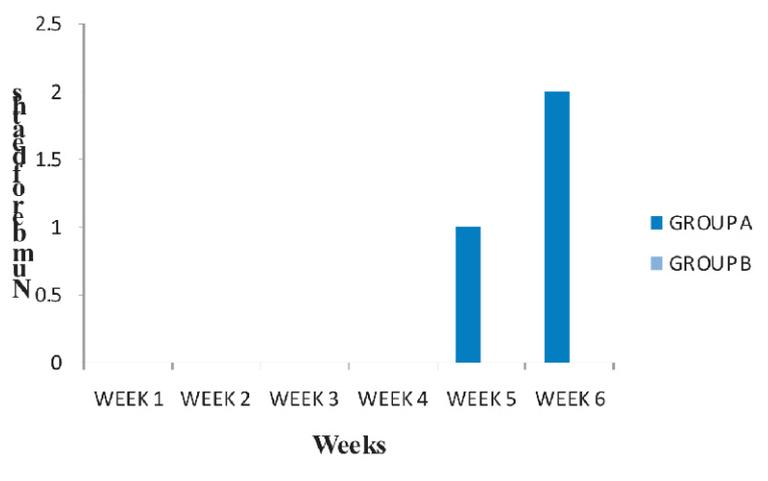**Group B**: Rats that received distilled water (Control)

Figure 3: Effects of MNU on the survival rate of experimental rats

KEY: Group A: Rats that received 100mg/kg/wt of MNU
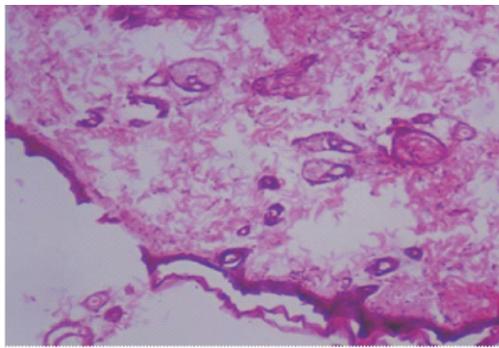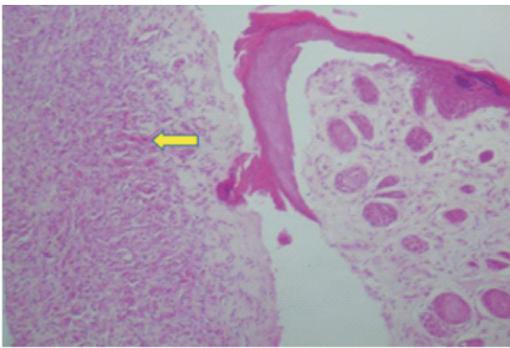**Group B**: Rats that received distilled water (Control)

PLATE 1A (HE − 100X) (severe panniculitis) PLATE 1B (HE − 100X)
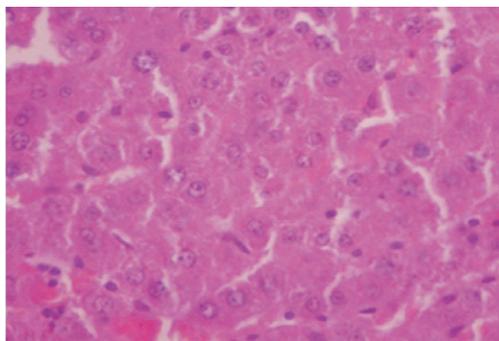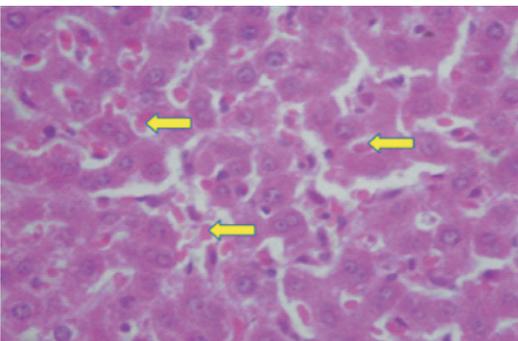**PLATE 1:** Histological sections of skin tissues of experimental rats

PLATE 2A (HE − 100X) (sinusoidal congestion) PLATE 2B (HE − 100X)
PLATE 2: Histological sections of liver tissues of experimental rats

Plate 3A shows the histologic sections of lung tissue with severe pulmonary inflammation. There is marked reduction in air filled alveolar spaces replaced by diffuse dense aggregates of inflammatory cell infiltrates while Plate 2B shows a histologic sections of a normal lung tissue showing air filled alveolar spaces with minimal surrounding interstitial inflammation or congestion.

The histologic section of breast tissue of experimental rats is shown in Plate 4A (rats induced with MNU) and Plate 4B (Control). Plate 4A shows absence of inflammatory cell infiltrates within the underlying dermis and subcutaneous fat of skin, however, an increase in fibrous tissue deposition is seen showing stromal fibrosis. Plate 4B histologic section of skin shows absence of inflammatory cell infiltrates within the underlying dermis and subcutaneous fat.
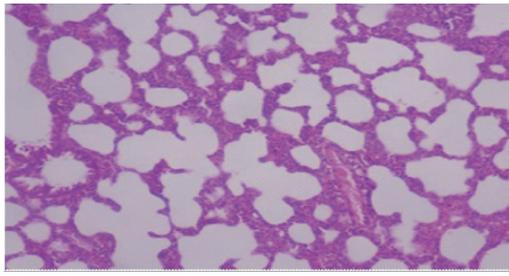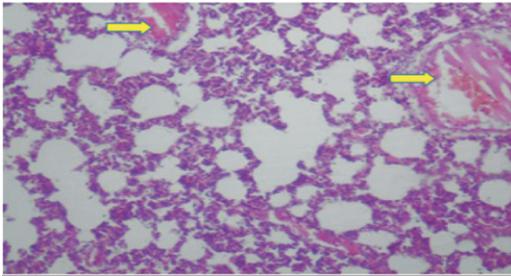
69

PLATE 3A (HE – 100X) PLATE 3B (HE – 100X)
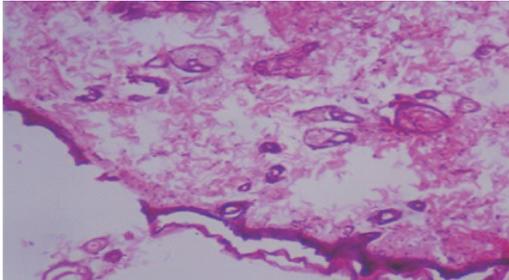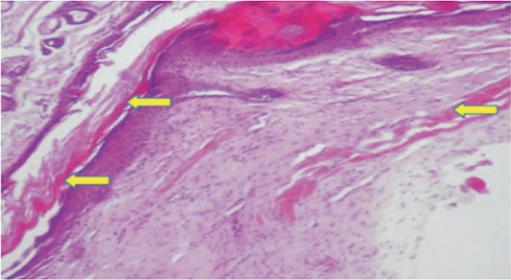**PLATE 3:** Histological sections of lung tissues of experimental rats

PLATE 4A (HE – 100X) (Stromal fibrosis) PLATE 4A (HE – 100X)
PLATE 4: Histological sections of breast tissues of experimental rats

Key:PLATE A: Rats that received 100mg/kg/wt of MNU
PLATE B: Rats that received distilled water (Control)

Discussion

Methylnitrosourea (MNU), a highly reactive chemical generated in certain foods can introduce alkyl radicals (methyl group specifically) into biologically active molecules such as DNA and thereby prevent their proper functioning ((NJDHSS, 2017). Many are used as antineoplastic agents, but most are very toxic, with carcinogenic, mutagenic, teratogenic, and immunosuppressant actions. Its carcinogenic effect was evaluated in the present study (IARC, 2017).

Results indicated increase in the average body weight of the rats during the study. Although the rats in groups A (Table 1) had the lowest increase in average body weight which could be attributed to the toxicity of MNU, but no statistically significant differences were observed (p > 0.05).There were also no significant changes between the weights of different organs (liver, kidney, lungs, spleen and heart) of rats in two different groups in the present study (data not shown). The shorter duration of exposure when compared to literature, used in this study might have prevented the observation of statistically significant difference in average weight.

The high death rate recorded in group A

(Figure 3) might be a consequence of the modified dosage which involved dosing of animals with 100 mg/kg/bwt of MNU against 50mg/kg/bwt used in studies documented in literature.

Serum tumour markers results from cancer antigen 15.3 (CA 15.3), carcinoembryonic antigen (CEA), and cancer antigen 27.29 (CA 27.29), showed a trend typical of their usage in monitoring breast cancer. CA 15.3 results gave higher significant difference compared to CEA (p < 0.05) while CA 27.29 showed no significant difference. These observations affirm the fact that CA 15.3 is most widely used serum markers in breast cancer studies followed by CEA while CA 27.29 list among the less widely used markers including tissue polypeptide antigen (TPA), tissue polypeptide specific antigen (TPS) and the shed form of HER-2 (François-Clément et al., 2012).These serum markers were ranked based on the following potentials:- early diagnosis, determining prognosis, prospectively predicting response or resistance to specific therapies, surveillance after primary surgery, and monitoring therapy in patients with advanced disease (Eghdami et al., 2014).

Severe panniculitis seen in group A (Plate 1A) describes inflammation of the subcutaneous fat that can result from multiple causes. In this study the occurrence of panniculitis could be suggested to be as a result of the MNU administered. Panniculitis can be classified as lobular or septal depending on the principal site of the inflammation within the fat (Gonzalez, 2017). The panniculitis as seen above could be an early stage of sclerosing panniculitis, in which there is sparse inflammatory infiltrate mostly composed of lymphocytes between the collagen bundles of the septa (Requena, and Sánchez, 2001).

Sinusoidal congestion seen in Plate 2A is in tandem with recent study which revealed the occurrence of non-tumorous liver parenchyma cells showing diffuse sinusoidal dilatation and congestion with extravasation of red blood cells which emanated from oxaliplatin-based chemotherapy (Seo and Kim, 2014). This histologic revelation follows a proven fact that, though, MNU which was originally designed as a chemotherapeutic alkylating compound could exert direct carcinogenic effects (Tsubura et al., 2011). Sinusoidal obstruction syndrome related to oxaliplatin administration which was first described by Rubbia-Brandt et al., 2005, and reported by Tsubura et al., 2011, is characterized by various histologic findings which included sinusoidal dilatation and congestion, which are generally irregularly distributed within the hepatic parenchyme.

Clinical and epidemiologic studies have suggested a strong association between severe pulmonary inflammation and cancer. Inflammation is a critical component of tumour progression (Valavanidis et al., 2013). The inflammatory component in the development of the neoplasm includes a diverse leukocyte population; these components are considered inflammatory tumour key factors promoting tumour progression due to their ability to release a variety of cytokines, chemokines, and cytotoxic mediators such as reactive oxygen species (ROS), metalloproteinases, interleukins, and interferons (Gomes et al., 2014). Cancer-related inflammation affects many aspects of malignancy, including the proliferation and survival of malignant cells, angiogenesis, tumour metastasis, and tumour response to chemotherapeutic drugs and hormones. Owing to the foregoing, the severe pulmonary inflammation seen in about ninety percent of rats in group A (Plate 3A) could be suggested to result from the carcinogenic effect of MNU. Cancer-associated inflammation has been linked with immune-suppression that allows cancer cells to evade detection by the immune system. Many cancers arise from sites of infection, chronic irritation and inflammation. It is now becoming clear that the tumour microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, fostering proliferation, survival and migration. Chronic inflammation is associated with angiogenesis, a hallmark of cancer and various ischaemic and inflammatory diseases (Valavanidis et al., 2013).

The occurrence of stromal fibrosis in the breast tissue of group A (Plate 4A) could be a resultant carcinogenic effect of MNU administered. Stromal fibrosis is a histopathology diagnosis characterized by proliferation of hypocellular fibrous tissue with the obliteration or hypoplasia of mammary lobules and ducts (Malik et al., 2014). In humans, stromal fibrosis is a common finding on percutaneous breast biopsy, with an incidence ranging from 2.1% to 9.0% depending on the series. Although the cause of stromal fibrosis has not yet been fully elucidated, it has been observed that stromal fibrosis can occur as a desmoplastic response to malignancy (Malik et al., 2014). Stromal fibrosis has been described by a variety of terms including "focal fibrous disease of the breast," "fibrosis of the breast," "fibrous mastopathy," "fibrous tumour of the breast," and "focal fibrosis" of the breast (Lee et al., 2011). Stromal fibrosis may present as a palpable discrete mass at both mammography and sonography, or as a clinically occult, imaging-detected abnormality (Sklair-Levy et al., 2001; Lee et al., 2011). The organs selected in this study were chosen based on reports of the effects MNU on experimental rats and mice. This result confirms the reason for the popular use of this experimental model in the investigation of breast cancer and its mimicry to human breast cancer (Ashrafi et al., 2012). Different forms of early stages of carcinogenesis was induced in female Sprague-Dawley rats using a novel and modified cancer induction protocol, including four injections of 100 mg/kg/bwt dosage of MNU beginning from 30 days of the rat's age and continued with a 7day interval. Knowledge from

this study did not only provide insight into possible harmful effects of MNU which could be obtained from foods containing nitrosamines, but it also provided the opportunity to test and prove a modified protocol of cancer induction which could be used to evaluate the preventive and therapeutic effect of different agents for human breast cancer within a short period of time.

REFERENCES

Ashrafi, M., Bathaie, S. Z. and Abroun, S. (2012). High expression of cyclin D1 and p21 in N-Nitroso-N-Methylurea-induced breast cancer in Wistar albino female rats. Cell Journal. 14(3): 193 – 202.

Chan, M. M., Lu, X., Merchant, F. M., Iglehart, J.D. and Miron, P. L. (2005). Gene expression profiling of NMU-induced rat mammary tumors: cross species comparison with human breast cancer. Carcinogenesis. 26(8): 1343 – 1353.

Chena,T., Taob, Y., Yana, W., Yanga, G., Chena, X., Caoa, R., Zhanga, L., Xuea, J. and Zhang, Z. (2016). Protective effects of hydrogen-rich saline against N-methyl-N-nitrosourea-induced photoreceptor degeneration. Exp. Eye Res. 148: 65 – 73.

Chinedu, E., Arome, D., and Ameh, F. S. (2013). A new method for determining acute toxicity in animal models. Toxicol. Int. 20(3): 224 – 226.

Eghdami, A., Mehdi, S. and Sohi, H. (2014). Investigation of α-IFN –SWNT and α-IFN-PLGA effects on breast cancer in rats induced by DMBA by using CA15-3 tumor marker. Adv. Biores. 5(2): 9 – 13.

François-Clément, B., Hajage, D., Bachelot, T., Delaloge, S. and Brain, E. (2012). Assessment of circulating tumour cells and serum markers for progression-free survival prediction in metastatic breast cancer: a prospective observational study. Breast Cancer Res. 14(1): 19 – 24.

Gomes, M., Teixeira, A. L., Coelho, A., Araújo, A. and Medeiros, R. (2014). The role of inflammation in lung cancer. In: Aggarwal, B., Sung, B., Gupta, S. (eds) Inflammation and Cancer. Springer International Publishing AG. Accessed 13th August, 2017.<https://link.springer.com/chapter/10.1007/978-3-0348-0837-8_1>

Gonzalez, M. E. (2017). Miller School of Medicine, University of Miami. Accessed 13th August, 2017. <http://www.msdmanuals.com/professional/dermatologic-disorders/hypersensitivity-and-inflammatory-skin-disorders/panniculitis>

Guru, K. D., Parvathi, V., Meenakshi, P., Rathi, M. A. and Gopalakrishnan, V. K. (2013). Anticancer activity of the ethanolic extract of Cratevanurvala bark against testosterone and MNU-induced prostate cancer in rats. Chin. J. Nat. Med. 10: 334 – 338.

International Agency for Research on Cancer (IARC). 2017. Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Geneva: World Health Organization, International Agency for Research on Cancer. Accessed 13th August, 2017. <http://monographs.iarc.fr/ENG/Classification/index.php>

Klug, W. S., Cummings, M. R., Spencer, C. A. and Palladino, M. A. 2015. Essentials of genetics. 11th ed., Pearson Education Inc. San Francisco. 508pp.

Lee, S., Mahoney, M. C., and Khan, S. (2011). MRI features of stromal fibrosis of the breast with histopathologic correlation. American Roentgen Ray Society. Accessed 10th September, 2017.<http://www.ajronline.org/doi/pdf/10.2214/AJR.11.6489>

Leung, W. K., Wu, K. C., Wong, C. Y., Cheng, A. S., Ching, A. K., Chan, A. W., Chong, W. W., Go, M. Y., Yu, J., To, K. F., Wang, X., Chui, Y. L., Fan, D. M. and Sung, J. J. (2008). Transgenic cyclooxygenase-2 expression and high salt enhanced susceptibility to chemical-induced gastric cancer development in mice. Carcinogenesis. 29: 1648 – 1654.

Malik, N., Lad, S., Seely, J. M. and Schweitzer, M. E. (2014). Underestimation of malignancy in biopsy-proven cases of stromal fibrosis. Brit. J. Radiol. 87: 1 – 6.

National Center for Biotechnology Information. (NCBI). (2017). PubChem Compound Database. Accessed 12th October, 2017.<https://pubchem.ncbi.nlm.nih.gov/comp ound/N-Methyl-N-nitrosourea#section=Top>

New Jersey Department of Health and Senior Services. (2016). Hazard substance fact sheet. Accessed 30th July, 2017. 6pp. <http://nj.gov/health/eoh/rtkweb/documents/f s/1411.pdf>

Parasuraman, S., Raveendran, R., and Kesavan, R. (2010). Blood sample collection in small laboratory animals. J. Pharmacol. Pharmacother. 1(2): 87–93.

Pula, B., Malicka, I., Pawlowska, K., Paslawska, U., Cegielski, M., Podhorska-Okolow, M., Dziegiel, P. and Wozniewski, M. (2013). Immunohistochemical characterization of N-methyl- N-nitrosourea-induced mammary tumours of Sprague- Dawley rats. In vivo. 27: 793 – 801.

Requena, L. and Sánchez Yus E. (2001). Panniculitis. Part II. mostly lobular panniculitis. American Academy of Dermatology, Inc. Accessed 5th August, 2017. <http://www.jaad.org/article/S0190-9622(01)79424-8/pdf>

Rubbia-Brandt, L., Audard, V., Sartoretti, P., Roth, A. D., Brezault, C. and Le Charpentier, M. (2004). Severe hepatic sinusoidal obstruction associated with oxaliplatin-based chemotherapy in patients with metastatic colorectal cancer. Ann. Oncol. 15: 460 – 466.

Sajjadi, M. and Bathaie, Z. (2016). Comparative study on the preventive effect of Saffron, Carotenoids, Crocin and Crocetin, in NMU-Induced breast cancer in rats. Cell Journal. 19(1): 95 – 101.

Saminathan, M., Rai, R. B., Dhama, K., anganath, G. J., Murugesan, V., Kannan, K., Pavulraj, S. and

Gopalakrishnan, A. and Suresh, C. (2014). Histopathology and immunohistochemical expression of N-Methyl-N-Nitrosourea (NMU) induced mammary tumours in Sprague-Dawley rats. Asian J. Anim.Vet. Adv. 9: 621 – 640.

Seo A. N. and Kim, H. (2014). Sinusoidal obstruction syndrome after oxaliplatin-based chemotherapy. Clin. Mol. Hepatol. 20: 81 – 84.

Sklair-Levy, M., Samuels, T. H., Catzavelos, C., Hamilton, P. and Shumak, R. (2001). Stromal fibrosis of the breast. American Roentgen Ray Society. Accessed 10th August, 2017.<http://www.ajronline.org/doi/pdf/10.22 14/ajr.177.3.1770573>

Takayama, S., Thorgeirsson, U. P. and Adamson, R. H. (2008). Chemical carcinogenesis studies in non-human primates. P Jpn Acad B Phys. 84: 176 – 188.

Tsubura, A., Lai, Y. C, Miki, H., Sasaki, T., Uehara, N., Yuri, T. and Yoshizawa, K. 2011. Review: Animal models of N-Methyl-N-nitrosourea-induced mammary cancer and retinal degeneration with special emphasis on therapeutic trials. In Vivo. 25: 11 – 22.

Valavanidis, A., Vlachogianni, T., Fiotakis, K., and Loridas, S. (2013). Pulmonary oxidative stress, inflammation and cancer: respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms. Int J Environ. Res. Public Health. 10: 3886 – 3907.

Xiong, Y., Ji, H., Song, W., Yin, Y., Xia, C., Xu, B., Xu, Y. and Xia, X. (2016). N-methyl-N-nitrosourea induces retinal degeneration in the rat via the inhibition of NF-κB activation. Cell Biochem. Funct. doi:10.1002/cbf.3232

Yuri, T., Danbara, N., Tsujita-Kyutoku, M., Fukunaga, K., Takada, H., Inoue, Y., Hada, T. and Tsubura, K. (2003). A dietary docosahexaenoic acid suppresses N-methyl N-nitrosourea-induced mammary carcinogenesis in rats more effectively than eicosapentaenoic acid. Nutr. Cancer. 45: 211 – 217.