Short Communication

Susceptibility to *N*-methyl-*N*-nitrosourea-induced retinal degeneration in different rat strains

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Abstract: To evaluate the potential role of genetic background in the susceptibility to retinal degeneration induced by *N*-methyl-*N*-nitrosourea (MNU), female rats of the Sprague-Dawley (SD), Long-Evans (LE) and Copenhagen (CH) strains were administered 50 mg/kg MNU or saline at 7 weeks of age. Retina morphology and morphometric analysis of all rats was performed 7 days after MNU administration. Atrophy of both the peripheral and central outer retina occurred in all rat strains exposed to MNU. Decreased photoreceptor cell ratio and increased retinal damage ratio were observed. The severities of the retinal atrophy were similar among all three rat strains. In conclusion, MNU-induced photoreceptor degeneration developed consistently in all three strains regardless of the absence (SD rats) or presence (LE and CH rats) of melanin in the retina, suggesting that genetic and melanin factors did not affect photoreceptor cell death after MNU. (DOI: 10.1293/tox.2015-0062; J Toxicol Pathol 2016; 29: 67–71)

Key words: *N*-methyl-*N*-nitrosourea, rats, retinal degeneration, strain difference

VISION 2020, which is supported by the WHO Programme for the Prevention of Blindness, is currently attempting to eliminate the main causes of blindness by 2020 in order to ensure all people in the world have the right to sight, particularly the millions who will needlessly become blind[1, 2]. Retinitis pigmentosa (RP) is one of the most common forms of inherited blindness worldwide; the pathology begins with early night blindness followed by peripheral visual field alterations (tunnel vision) and eventually complete blindness[3, 4]. Animal models of retinal degeneration have been shown to be important tools for elucidating the mechanisms of human blindness and exploring potential treatments. *N*-methyl-*N*-nitrosourea (MNU) is an alkylating agent that targets photoreceptor cells, thereby rapidly inducing retinal degeneration via apoptosis in rats, in a manner that is similar to the mechanism of human RP[5, 6]. The MNU-induced apoptosis cascade involves upregulation of the Bax protein, down-modulation of the Bcl-2 protein and activation of the caspase family proteins. Within one week after exposure to an adequate dose of MNU, active signs of photoreceptor degeneration become distinct due to the loss of photoreceptor cells.

It is known that there are racial differences in the prevalence of certain human retinal degenerative diseases, such as age-related macular degeneration[7]. Also, the genetic background in rodents has been reported to affect the biological reactions of the eye, such as photoreceptor degeneration induced by light[8], hypoxia and hyperoxia[9] and retinal detachment[10]. Susceptibility to MNU-induced carcinogenesis in certain organs differs among rat strains[11-13]. Thus, differential susceptibility to MNU-induced neurodegeneration may be an important factor. However, there have been no reports concerning the strain difference in MNU-induced retinal damage in rats. In the present study, we evaluated the potential of rat genetic backgrounds to affect MNU-induced retinal degeneration.

The experimental protocol and all animal procedures used in this study were approved by the Animal Care and Use Committee of Kansai Medical University and were in accordance with the guidelines for animal experimentation at Kansai Medical University. Animals were housed in plastic cages with paper-chip bedding (Paper Clean; Japan SLC, Hamamatsu, Japan) in an air-conditioned room maintained at $22 \pm 2°C$ and $60 \pm 10\%$ relative humidity with a 12 hr light/dark cycle. The illumination intensity was below 60 lux in the cages. Seven-week-old Sprague-Dawley (SD) rats [Slc:CD], Long-Evans (LE) rats [Iar:Long-Evans] and Copenhagen (CH) rats [Iar:Copenhagen] (each strain n=10) were purchased from Japan SLC. Rats were maintained in

specific pathogen-free conditions and had free access to water and a commercial pellet diet (CMF 30 kGy; Oriental Yeast, Chiba, Japan).

MNU was obtained from Sigma-Aldrich (St. Louis, MO, USA) and stored at −80°C in the dark. The MNU solution was dissolved in physiologic saline containing 0.1% acetic acid just prior to use. At 7 weeks of age, MNU (50 mg/kg) or vehicle (physiological saline) was administered to 5 rats of each strain once by the intraperitoneal (ip) route. Rats were sacrificed 7 days after MNU administration. The experimental protocol used was similar to our previous report[14]. At the time of sacrifice, both eyes were quickly removed from each animal, and each eye was fixed overnight in methacarn (60% methanol, 30% chloroform and 10% acetic acid). The eyes were cut along a line parallel to the optic axis and nerve (including the ora serrata), embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin (HE). All systemic organs were also fixed overnight in 10% neutral buffered formalin for histopathological examination.

HE-stained eye sections were scanned with a high-resolution digital slide scanner (NanoZoomer 2.0 Digital slide scanner; Hamamatsu Photonics, Hamamatsu, Japan) to obtain digital images that were opened in color mode using the NDP.view software (Hamamatsu Photonics). Total retinal thickness (from the internal limiting membrane to the pigment epithelium) and outer retinal thickness (from the outer nuclear layer to the pigment epithelial cell layer) were individually measured from the HE-stained slides using NDP.view, as previously described[5, 6]. To further evaluate photoreceptor cell loss, we calculated the photoreceptor ratio [(outer retinal thickness / total retinal thickness) × 100].

To determine the area of retinal damage, the entire length of the retina and the length of the damaged area in the HE preparations were measured. Damaged retina was defined as the presence of less than four rows of photoreceptor nuclei in the outer nuclear layer[5, 6], with the retinal damage ratio calculated as follows: [(length of damaged retina / entire retinal length) × 100]. Two toxicologic pathologists, (K.Y., A.T.) certified by the Japanese Society of Toxicologic Pathology performed histopathological evaluations according to previously defined histopathological terminology and diagnostic criteria[5, 6].

All discrete values were expressed as the mean ± standard error (SE). After confirming the homogeneity of the variances, data were analyzed using a two-tailed independent Student's *t*-test for unpaired samples (Excel 2007®, Microsoft, Redmond, WA, USA). The results presented below include comparisons between the saline- and MNU-treated rats in each strain, and among MNU-treated rats in each strain. *P* values < 0.05 were considered to be statistically significant.

No deaths occurred in any strain with or without MNU treatment during the study period. However, body weight gain was significantly suppressed in all strains of MNU-exposed rats compared with saline-treated rats groups; 41.6 ± 6.9 and 25.7 ± 4.6 g in saline- and MNU-treated SD rats,

44.7 ± 4.7 and 19.9 ± 3.1 g in saline- and MNU-treated LE rats and 29.6 ± 3.0 and 12.5 ± 2.4 g in saline- and MNU-treated CH rats, respectively. The decreases in body weight ([100 - (body weight before MNU exposure / body weight at sacrifice) × 100]) observed were 38.8% in SD rats, 55.5% in LE rats and 57.8% in CH rats, respectively. Therefore, there was no strain difference in the effects on body weight change after MNU exposure.

In all rat strains 7 days after saline treatment, no abnormal histopathological changes in the central and peripheral retina were observed. In all rats, the retinas contained more than ten layers of photoreceptor nuclei in the central retina (Fig. 1a, 1c, and 1e) and more than eight layers of cells in the peripheral retina (data not shown). At 7 days after a single MNU injection in all rat strains, the outer nuclear layer and the photoreceptor layer in the central and peripheral retina either disappeared or were reduced to a few rows of photoreceptor cell nuclei (Fig. 1b, 1d, and 1f). In the damaged photoreceptor layer of LE and CH rats (with melanin in the pigment epithelial layer), melanophages (macrophages that phagocytosed melanin) were observed, suggesting reactive changes of the pigmented epithelial cells (Fig. 1d and 1f), as well as the hypertrophic pigmented epithelial cells in SD rats (Fig. 1b). To further evaluate the susceptibility to the effects on retinal thickness at the central retina, the photoreceptor cell ratio was calculated. There were statistically significant changes in the photoreceptor cell ratios in the peripheral and central retina between the saline-treated and MNU-treated rats, with the ratios in the peripheral retina being 46.4 ± 3.6% and 24.9 ± 1.0% in saline- and MNU-treated SD rats, 48.9 ± 2.3% and 28.1 ± 9.9% in saline- and MNU-treated LE rats, and 52.9 ± 2.4% and 33.2 ± 5.4% in saline- and MNU-treated CH rats and the ratios in the central retina being 46.0 ± 2.0% and 12.6 ± 1.8% in saline- and MNU-treated SD rats, 46.7 ± 2.2% and 14.7 ± 2.1% in saline- and MNU-treated LE rats and 50.0 ± 1.2% and 15.3 ± 0.4% in saline- and MNU-treated CH rats, respectively (Fig. 2a). However, there were no statistically significant changes in the photoreceptor cell ratios at the peripheral and central retina among the MNU-treated SD, LE and CH rats. In addition, the MNU-treated rats of all three strains exhibited statistically significant changes in the retinal damage ratio compared with the saline-treated groups (0.0 in saline-treated rats of each strain and 89.7 ± 12.7, 93.5 ± 5.6 and 95.6 ± 2.7 in MNU-treated rats of the SD, LE and CH strains, respectively) (Fig. 2b). Again, there were no statistically significant differences in retinal damage ratios among the MNU-treated SD, LE and CH rats. Careful histopathological observation of the systemic organs revealed the presence of lymphohematopoietic toxicity, including decreased cellularity in the bone marrow, and atrophy of the thymus, splenic white pulp and mesenteric lymph nodes in MNU-treated all rat strains. The degrees of these lesions were not different among the three strains.

From a toxicological perspective, strain differences give an indication of the effect of genetic variation on responses to toxins, and chemical safety can be assessed using
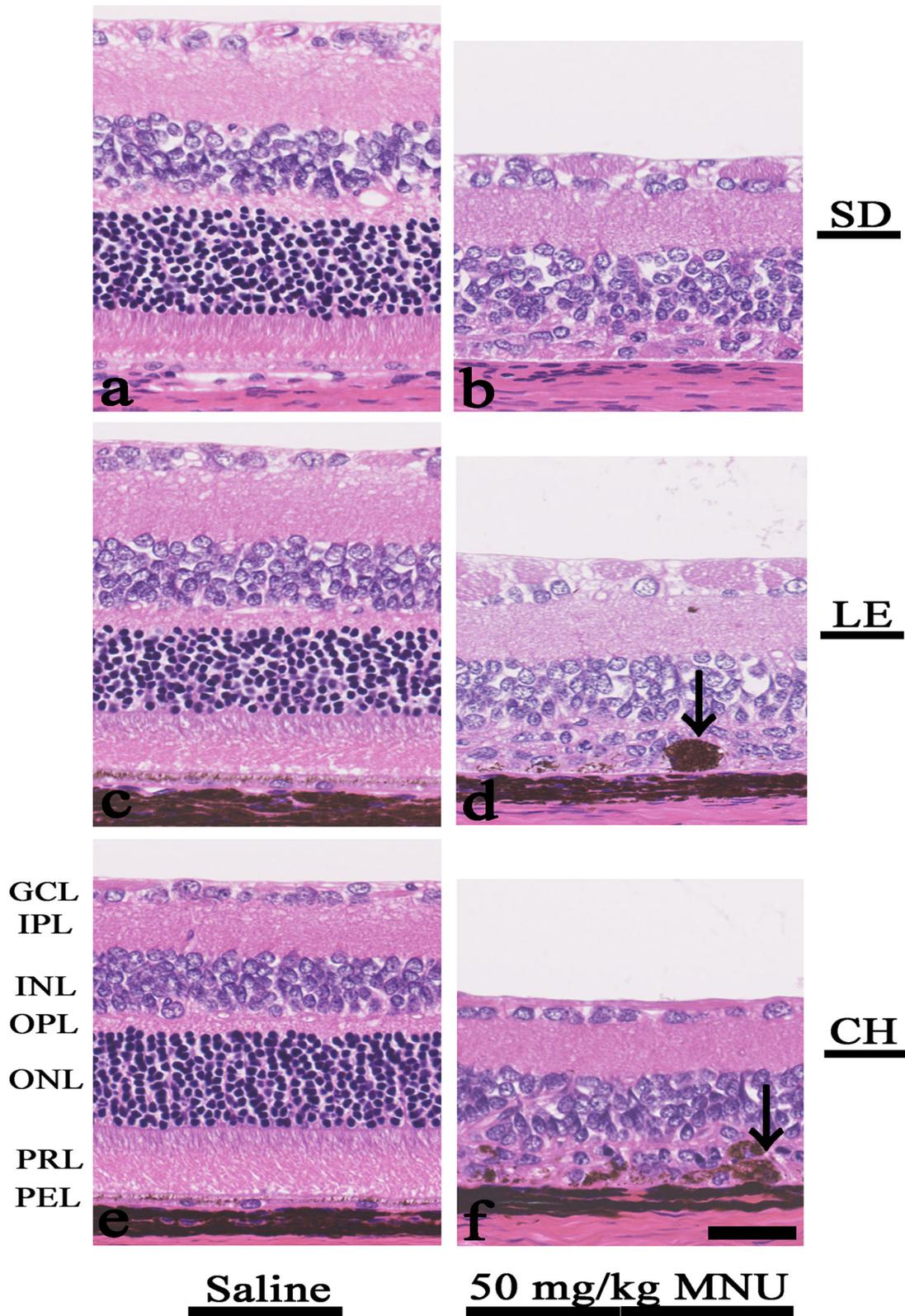
Fig. 1. Histology of the central retina in Sprague Dawley (SD), Long-Evan (LE) and Copenhagen (CH) rats treated with saline or 50 mg/kg MNU. At 7 days after a single ip injection of MNU, the outer nuclear layer and photoreceptor layer disappeared in all rat strains. (a) Saline-treated SD rat, which lacks melanin in the retina. (b) MNU-treated SD rat. (c) Saline-treated LE rat, which has melanin in the retina. (d) MNU-treated LE rat. (e) Saline-treated CH rat, which has melanin in the retina. (f) MNU-treated CH rat. Note the presence of melanophage cells, which phagocytized melanin (arrows in d and f). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photoreceptor cell layer; and PEL, pigment epithelial cell layer. HE staining, bar = 50 μm.
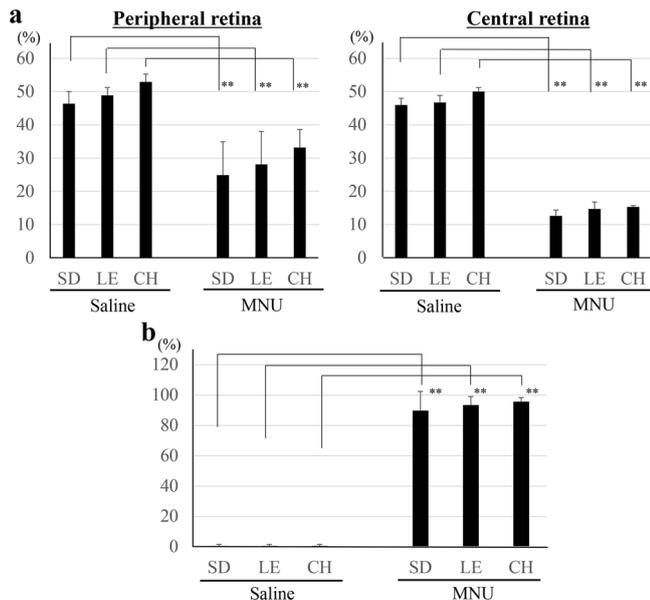
Fig. 2. Morphometric analysis of the retina in Sprague Dawley (SD), Long-Evan (LE) and Copenhagen (CH) rats treated with saline or 50 mg/kg MNU. (a) Photoreceptor cell ratios in the central and peripheral retina 7 days after a single ip injection of MNU. Rats of all strains treated with 50 mg/kg MNU exhibited statistically significant changes in their photoreceptor ratios at the central and peripheral retina compared with the saline-treated control group. However, there was no significant changes in photoreceptor ratios at the central and peripheral retina among the MNU-treated SD, LE and CH rats. The index was calculated as [(outer retinal thickness / total retinal thickness) × 100]. (b) Retinal damage ratio in MNU-treated rats. MNU-treated rats of all strains show statistically significant changes in the retinal damage ratio, compared to the saline-treated control group. However, no significant changes were detected among MNU-treated SD, LE, and CH rats. The index was evaluated as [(length of retina composed of less than four photoreceptor cells / whole retinal length) × 100]. The mean ± SE of five rats in each treatment group is shown. **$p < 0.01$.

the most sensitive strain[15]. Further investigation could lead to the identification of susceptibility or resistance genes, a better understanding of mechanisms and possibly therapeutic targets. Laboratory rats and mice have a wide range of susceptibilities to xenobiotics and carcinogenes[15]. In the present study, atrophy of the outer retina, at both the peripheral and central retina, occurred in MNU-treated rats of the SD, LE and CH strains, and the severities of retinal atrophy were similar among all three strains. Retinal morphometrical analysis, such as for the photoreceptor cell ratio and retinal damage ratio, did not reveal any statistically significant differences among the three rat strains.

MNU is a methylated nitrosourea compound with alkylating, mutagenic, teratogenic, carcinogenic and cytotoxic properties[16]. The MNU-induced mammary cancer rat model is widely used to screen and evaluate the potency of cancer- suppressing/promoting agents[17, 18]. Different strains of rodents often have different levels of susceptibility and resistance to the chemical induction of cancer in spe-

cific tissues. A number of rat strains, such as SD rats, are highly susceptible to MNU-induction of mammary carcinogenesis[11, 19], while LE and CH rats are not highly susceptible[11, 12, 19, 20], probably due to differences in their genetic backgrounds. However, in the present study, SD, LE and CH retinas were equally susceptible to MNU. In general, the retinal pigment epithelium affects the biological reactions of the eye, such as in light-induced photoreceptor degeneration[8]; nonpigmented rats, which do not possess melanin in the retinal pigment epithelial layer, are highly susceptible to light-induced changes[21]. Many chemicals and drugs are known to bind to ocular melanin (choroid and pigment epithelial cells), and binding of drugs to melanin is known to affect the pharmacologic and pharmacokinetic properties of chemical molecules in pigmented rats that possess retinal melanin[22]. In pigmented rats, it is therefore possible that retinal toxicity results directly from the high concentration of chemicals sequestered in the pigment epithelial cells by virtue of chemical binding to melanin[23, 24]. In agreement with a previous study in which Brown-Norway rats were exposed to 75 mg/kg MNU[25], MNU-induced retinal degeneration in rats does not appear to depend on the presence of ocular melanin. Similar to SD rats, LE rats are sensitive to retinal degeneration induced by 75 mg/kg MNU[26]. There are no reports in the literature that have focused on chemical structure and molecular characterization in an attempt to indicate that MNU binds melanin[27].

In conclusion, susceptibility to MNU-induced photoreceptor degeneration in rats was not significantly different among the three strains examined, regardless of the presence of melanin in the retina. Therefore, there is no evidence indicating that genetic factors affect MNU-induced photoreceptor cell death.

References

1. Resnikoff S, and Pararajasegaram R. Blindness prevention programmes: past, present, and future. Bull World Health Organ. **79**: 222–226. 2001. [Medline]
2. World Health Organization (WHO), Prevention of Blindness and Deafness Programme Global Data on Visual Impairments 2010. 2012. Retrieved July 15, 2014, from World

Health Organization website: http://www.who.int/blindness/GLOBALDATAFINALforweb.pdf.

3. Hartong DT, Berson EL, and Dryja TP. Retinitis pigmentosa. Lancet. **368**: 1795–1809. 2006. [Medline] [CrossRef]

4. Davis RJ, Hsu CW, Tsai YT, Wert KJ, Sancho-Pelluz J, Lin CS, and Tsang SH. Therapeutic margins in a novel preclinical model of retinitis pigmentosa. J Neurosci. **33**: 13475–13483. 2013. [Medline] [CrossRef]

5. Yoshizawa K, and Tsubura A. Characteristics of *N*-methyl-*N*-nitrosourea-induced retinal degeneration in animals and application for the therapy of human retinitis pigmentosa. Nippon Ganka Gakkai Zasshi. **109**: 327–337. 2005; [Abstract in English and text in Japanese]. [Medline]

6. Yoshizawa K, Emoto Y, and Tsubura A. *N*-methyl-*N*-nitrosourea-induced retinal degeneration in rats: a reliable animal model of retinitis pigmentosa for the development of new therapeutic strategies. In: Recent Advances in Eye Research. A Hogarth, ed. Nova Science publishers, New York. 53–97, 2015.

7. Friedman DS, Katz J, Bressler NM, Rahmani B, and Tielsch JM. Racial differences in the prevalence of age-related macular degeneration: the Baltimore Eye Survey. Ophthalmology. **106**: 1049–1055. 1999. [Medline] [CrossRef]

8. LaVail MM, Gorrin GM, Repaci MA, and Yasumura D. Light-induced retinal degeneration in albino mice and rats: strain and species differences. Prog Clin Biol Res. **247**: 439–454. 1987. [Medline]

9. Walsh N, Bravo-Nuevo A, Geller S, and Stone J. Resistance of photoreceptors in the C57BL/6-c2J, C57BL/6J, and BALB/cJ mouse strains to oxygen stress: evidence of an oxygen phenotype. Curr Eye Res. **29**: 441–447. 2004. [Medline] [CrossRef]

10. Matsumoto H, Kataoka K, Tsoka P, Connor KM, Miller JW, and Vavvas DG. Strain difference in photoreceptor cell death after retinal detachment in mice. Invest Ophthalmol Vis Sci. **55**: 4165–4174. 2014. [Medline] [CrossRef]

11. Huggins CB, Ueda N, and Wiessler M. *N*-Nitroso-*N*-methylurea elicits mammary cancer in resistant and sensitive rat strains. Proc Natl Acad Sci USA. **78**: 1185–1188. 1981. [Medline] [CrossRef]

12. Korkola JE, and Archer MC. Resistance to mammary tumorigenesis in Copenhagen rats is associated with the loss of preneoplastic lesions. Carcinogenesis. **20**: 221–227. 1999. [Medline] [CrossRef]

13. Suckow MA, Wolter W, and Pollard M. Susceptibility of Lobund-Wistar x Copenhagen hybrid rats to autochthonous prostate carcinogenesis. Prostate. **64**: 203–208. 2005. [Medline] [CrossRef]

14. Emoto Y, Yoshizawa K, Hamazaki K, Kinoshita Y, Yuki M, Yuri T, Kawashima H, and Tsubura A. Mead acid supplementation does not rescue rats from cataract and retinal degeneration induced by *N*-methyl-*N*-nitrosourea. J Toxicol Pathol. **28**: 11–20. 2015. [Medline] [CrossRef]

15. Festing MFW. Inbred strains should replace outbred stocks in toxicology, safety testing, and drug development. Toxicol Pathol. **38**: 681–690. 2010. [Medline] [CrossRef]

16. Tsubura A, Yoshizawa K, and Sasaki T. Methylnitrosourea. In: Encyclopedia of Toxicology, 3rd ed., Vol. 3. P Wexler (ed). Academic Press/Elsevier, Oxford. 321–323. 2014.

17. Tsubura A, Lai YC, Miki H, Sasaki T, Uehara N, Yuri T, and Yoshizawa K. 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. 2011. [Medline]

18. Faustino-Rocha AI, Ferreira R, Oliveira PA, Gama A, and Ginja M. N-Methyl-N-nitrosourea as a mammary carcinogenic agent. Tumor Biol. Epub ahead of print. 2015.

19. Isaacs JT. Genetic control of resistance to chemically induced mammary adenocarcinogenesis in the rat. Cancer Res. **46**: 3958–3963. 1986. [Medline]

20. Isaacs JT. Inheritance of a genetic factor from the Copenhagen rat and the suppression of chemically induced mammary adenocarcinogenesis. Cancer Res. **48**: 2204–2213. 1988. [Medline]

21. Wasowicz M, Morice C, Ferrari P, Callebert J, and Versaux-Botteri C. Long-term effects of light damage on the retina of albino and pigmented rats. Invest Ophthalmol Vis Sci. **43**: 813–820. 2002. [Medline]

22. Durairaj C, Chastain JE, and Kompella UB. Intraocular distribution of melanin in human, monkey, rabbit, minipig and dog eyes. Exp Eye Res. **98**: 23–27. 2012. [Medline] [CrossRef]

23. Leblanc B, Jezequel S, Davies T, Hanton G, and Taradach C. Binding of drugs to eye melanin is not predictive of ocular toxicity. Regul Toxicol Pharmacol. **28**: 124–132. 1998. [Medline] [CrossRef]

24. Dayhaw-Barker P. Retinal pigment epithelium melanin and ocular toxicity. Int J Toxicol. **21**: 451–454. 2002. [Medline] [CrossRef]

25. Nakajima M, Yuge K, Senzaki H, Shikata N, Miki H, Uyama M, and Tsubura A. Photoreceptor apoptosis induced by a single systemic administration of *N*-methyl-*N*-nitrosourea in the rat retina. Am J Pathol. **148**: 631–641. 1996. [Medline]

26. Boudard DL, Mendoza J, and Hicks D. Loss of photic entrainment at low illuminances in rats with acute photoreceptor degeneration. Eur J Neurosci. **30**: 1527–1536. 2009. [Medline] [CrossRef]

27. Gnewuch CT, and Sosnovsky G. A critical appraisal of the evolution of *N*-nitrosourea as anticancer drugs. Chem Rev. **97**: 829–1014. 1997. [Medline] [CrossRef]