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Original Article

Histopathological changes in the olfactory epithelium of mice during four-week recovery after 2-ethyl-1-hexanol inhalation

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ABSTRACT — Volatile organic compound 2-ethyl-1-hexanol (2EH) causes the sick building syndrome. Inhalation exposure to 2EH causes olfactory epithelium (OE) degeneration and olfactory neuron loss in mice, which recover temporarily despite continued exposure but subsequently experience similar toxic effects. However, the exact course of recovery after 2EH cessation remains unknown. Therefore, in this study, we aimed to evaluate the histopathological changes in OE after 2EH inhalation cessation. Male ICR mice were exposed to 70 ppm 2EH for 8 hr daily, five days a week, for four weeks, followed by a recovery period of up to four weeks. Histopathological changes in mouse OE on the first (D1) and third (D3) days and first (W1), second (W2), and fourth (W4) weeks of the recovery period were analyzed. Notably, 2EH induced OE degeneration at W2, enlarged the Bowman's glands at W1 and W2, and decreased the olfactory marker protein-positive cell proportions at W1 and W2. Total leukocytes, neutrophils, and lymphocytes were abundant at both W1 and W2, with no significant differences. Proliferating cell nuclear antigen-positive basal cell number increased; they were distributed throughout the OE on D1 but subsequently lined up the basement membrane and remained at low levels thereafter. Number of growth-associated protein-43-positive immature olfactory neurons increased from D3 to W2. In conclusion, OE was not immediately repaired; the toxic effects appeared 1–2 weeks after 2EH cessation, as indicated by the OE tissue damage with decreased olfactory marker protein-positive cell proportions, followed by proper recovery.

Key words: 2-Ethyl-1-hexanol, Volatile organic compound (VOC), Inhalation exposure, Olfactory epithelium, Olfactory neuron, Histopathological changes

INTRODUCTION

Over the past two decades, 2-ethyl-1-hexanol (2EH; CAS number 104-76-7) has attracted attention as an inducer of sick building syndrome (Wieslander *et al.*, 1999; Kamijima *et al.*, 2002; Kamijima *et al.*, 2005; Wakayama *et al.*, 2019a). Emitted 2EH is generated by the hydrolysis of di(2-ethylhexyl) phthalate, a plasticizer in polyvinyl chloride flooring that reacts with the alkaline moisture in the building concrete (Kamijima *et al.*, 2005; Chino *et al.*, 2009). Its concentration is affected by temperature and humidity (Sakai *et al.*, 2009). Notably,

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high levels of 2EH in buildings in the summer can persist for over 10 years after construction (Wakayama *et al.*, 2019b). Therefore, inhalation exposure to 2EH and subsequent exposure cessation occur repeatedly over long periods.

Olfactory epithelium (OE) of the nasal cavity is directly damaged by exposure to various chemicals and constantly turned over to maintain the olfactory function (Harkema *et al.*, 2006). Additionally, OE rapidly regenerates and regains its function after tissue injury (Jang *et al.*, 2003). Histological changes in OE during inhalation exposure and subsequent recovery after exposure cessation to perchloroethylene, methyl methacrylate, methyl ethyl ketoxime, toluene, carbon dioxide, acetone, and pyridine have been demonstrated in experimental animals (Aoki *et al.*, 1994; Hext *et al.*, 2001; Newton *et al.*, 2002; Jacquot *et al.*, 2006; Buron *et al.*, 2009a; Buron *et al.*, 2009b; Buron *et al.*, 2013). Animal studies have shown that 2EH induces acute mucosal irritation (Scala and Burtis, 1973) and histopathological alterations in OE (Miyake *et al.*, 2016). Mice exposed to 20, 60, and 150 ppm 2EH via inhalation exhibit OE degeneration, loss of olfactory neurons, and neutrophil infiltration after one week but show repaired OE and increased olfactory neuron and proliferating basal cell numbers comparable to those in the control group one month after continuous 2EH exposure. After three months, re-degeneration of OE, lymphocyte infiltration, and decreased olfactory neurons are observed. This time course of OE changes is observed under continuous inhalation exposure for three months; however, the course of recovery after exposure cessation remains unknown. Therefore, we aimed to investigate the histopathological changes in OE during the four-week recovery period after cessation of inhalation exposure to 70 ppm 2EH.

MATERIALS AND METHODS

Animals and experimental design

This study protocol was approved by the Animal Care and Use Committee (approval numbers: H22M-72 and H28M-015) and adhered to the Guide for Animal Experimentation of Nagoya City University. Nine-week-old pathogen-free 30 male ICR mice were obtained from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan) and acclimatized for one week before the experiment. The overall experimental design, including the exposure and recovery periods, is shown in Fig. 1. The mice were randomly divided into two groups. Five mice in the control group were exposed to fresh air in a whole-body inhalation exposure chamber (Sibata Scientific Technology, Ltd., Saitama, Japan). The remaining twenty-five mice were exposed to 70 ppm 2EH for 8 hr daily, five days a week, for four weeks using the same inhalation exposure chamber system. The chamber was maintained under a 12-hr light/dark cycle at 23–24°C under relatively constant humidity (55%). Food and water were provided ad libitum. Subsequently, body weights of mice were measured once a week throughout the exposure period. After four weeks of inhalation exposure, the mice were removed from the exposure chamber for recovery. Five control mice (Con) were sacrificed on the first day of the recovery period, and five randomly selected mice were sacrificed on the first (D1) and third (D3) days and first

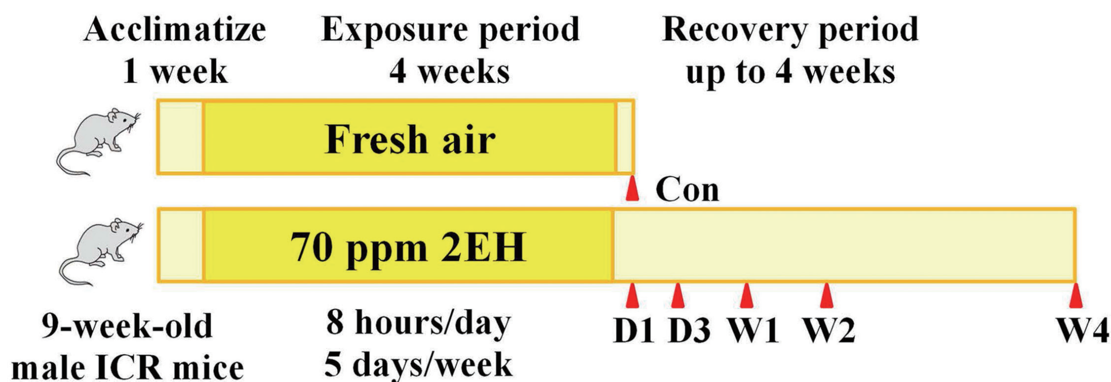

Fig. 1. Experimental design. Control mice were sacrificed on the first day of the recovery period (Con). Mice exposed to 70 ppm 2-ethyl-1-hexanol (2EH) were sacrificed on the first (D1) and third (D3) days and first (W1), second (W2), and fourth (W4) weeks of the recovery period. Red triangle indicates the sacrifice date. Each group consisted of five mice.

(W1), second (W2), and fourth (W4) weeks of the recovery period. The mice were weighed, deeply anesthetized, and transcardially perfused with 4% (w/v) paraformaldehyde phosphate buffer (Fujifilm Wako Pure Chemical Industries Ltd., Osaka, Japan) for histopathological examination. Finally, the mice were euthanized, and their nasal cavities were removed and fixed via immersion in 4% (w/v) paraformaldehyde.

Evaluation of 2EH concentration

Exposure concentration of 2EH was monitored via charcoal tube sampling (Sibata Scientific Technology Ltd.) at 1 L/min for 10 min once every two days using the MP-Σ300 mini pumps (Sibata Scientific Technology Ltd.). Then, 2EH was eluted from the absorbent with carbon disulfide and quantified via gas chromatography-mass spectrometry (Kamijima *et al.*, 2002). Average concentrations of 2EH in four weeks were 0 ppm (control group) and 71.6 ppm (exposure group). Air exchange rate in each chamber was 20 L/min.

Histological analyses

Nasal cavity was embedded in paraffin, sectioned, and stained as previously described (Miyake *et al.*, 2016). Subsequently, the sections were stained with hematoxylin (Mayer's Hematoxylin; Fujifilm Wako Pure Chemical Industries Ltd.) and eosin (Eosine Y; Muto Pure Chemicals Co. Ltd., Tokyo, Japan) and high iron diamine-Alcian blue (Iron(III) chloride, *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride, and *N*,*N*-dimethyl-1,3-phenylenediamine dihydrochloride [all from Sigma-Aldrich, MO, USA]; Alcian blue stain solution [pH 2.5; Muto Pure Chemicals Co. Ltd.]).

Olfactory, infiltrating, and other component cells in OE were immunostained with the appropriate antibodies and diaminobenzidine. The following primary antibodies were used: Rabbit anti-CD45 or CD3 polyclonal IgG (1:100; Abcam, Cambridge, UK), rabbit anti-neutrophil elastase (NE) polyclonal IgG (1:100; Calbiochem, Darmstadt, Germany), rabbit anti-olfactory marker protein (OMP) polyclonal IgG (1:200; Abcam), rabbit anti-growth associated protein (GAP)-43 monoclonal (1:1000; Abcam), and rabbit anti-proliferating cell nuclear antigen (PCNA) polyclonal IgG (1:50; Santa Cruz Biotechnology, Dallas, TX, USA) antibodies.

Quantitative analysis

Histological analyses were performed in five different microscopic fields at 400× magnification, as previously described (Miyake *et al.*, 2016). Morphological changes in OE after 2EH exposure were scored as 1 (normal), 2 (mild), 3 (moderate), or 4 (severe). Total score for each mouse was 1 (5–8 points/animal), 2 (9–12), 3 (13–16), or 4 (17–20). For Bowman's glands in the nasal mucosa, mean diameters (µm) of the glands in the lamina propria were determined, and the mean was calculated for the animals. CD45-, NE-, CD3-, GAP43-, and PCNA-positive cells were counted in each animal, and number of cells per mm^2 of OE was measured. Moreover, ratio of OMP-positive area to OE area was measured for each animal.

Data analysis

In this study, all statistical analyses were conducted using the EZR software package (version 1.41; Saitama Medical Center, Jichi Medical University, Saitama, Japan). Data were analyzed using the Kruskal–Wallis test followed by the Steel–Dwass post-hoc test or one-way analysis of variance followed by the Tukey honest significant difference post-hoc test. Statistical significance was set at $p < 0.05$.

RESULTS

Body weight

No deaths or signs of clinical abnormalities were observed in the control and 2EH-exposed groups during the exposure and recovery periods. Additionally, no significant differences in water and food consumption were observed between the exposure and recovery periods, and all mice consistently gained weight throughout the experimental period.

Morphological changes in OE

OE, lamina propria, and basement membrane (BM) were organized, and OE was uniformly covered with cilia in control mice (Fig. 2A). Morphology of OE in 2EH-exposed mice was similar to that of OE in control mice at D1 and D3. Number of cells in the OE decreased and the cells were disorganized at W1 and W2; however, the cells were reorganized at W4. Total score for all morphological changes in OE was significantly higher in the 2EH-exposed group than in the control group at W2 (Fig. 2A). Bowman's glands were significantly enlarged at W1 and W2 but returned to the control size at W4 (Fig. 2B).

Infiltration of leukocytes into OE

Exposure to 2EH induced leukocyte infiltration into OE; however, no infiltration was observed in the control group. At W1 and W2, total leukocytes (CD45-stained cells), neutrophils (NE-stained cells), and lymphocytes (CD3-stained cells), particularly neutrophils, were more

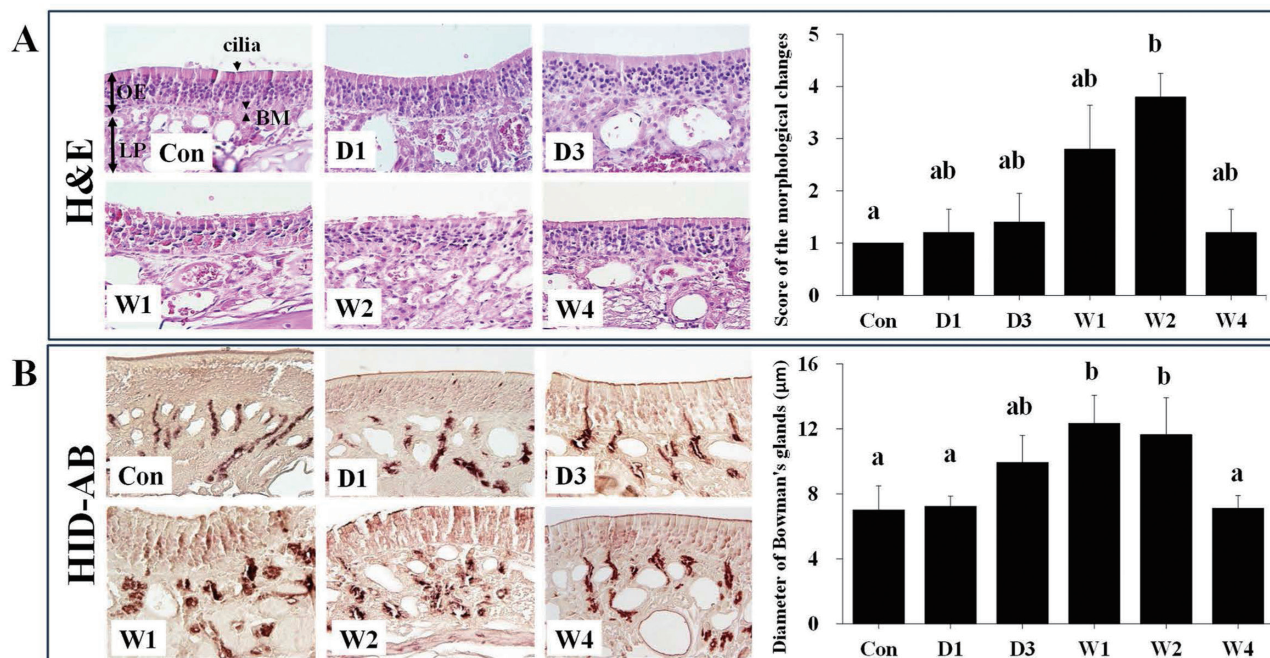

Fig. 2. Morphological changes in the olfactory epithelium (OE) of mice exposed to 2EH for four weeks, followed by recovery for 1 and 3 d and 1, 2, and 4 weeks. (A) OE was stained with hematoxylin and eosin (H&E) to assess the morphological changes in the nasal mucosa of mice. OE (black two-way arrow), LP (black two-way arrow), and BM (between the black arrowheads) indicate the olfactory epithelium, lamina propria, and basement membrane, respectively. Black arrowhead indicates the cilia. The figure on the right shows the total score rating of the observed morphological changes. (B) OE was stained with high iron diamine-Alcian blue (HID-AB) to observe the Bowman's glands. The figure on the right shows the diameter (µm) of Bowman's glands. Typical photographs of each group are shown. Scale bar, 50 µm. Each column and bar represent the mean and standard deviation (SD), respectively. Different letters above the bars indicate the statistically significant differences ($p < 0.05$; $n = 5$/group).

abundant in the 2EH-exposed group, but no significant differences were observed between both groups (Fig. 3).

Olfactory nerves in OE

Area of OMP-positive cells, indicating mature olfactory neurons (Menco, 1989), in OE continued to decrease until W2, showing a significant difference from the control group at W1 and W2, and subsequently recovered at W4 (Fig. 4A). GAP43 levels, indicating immature olfactory neurons (Bergman *et al.*, 2002), in OE at D3, W1, and W2 significantly increased in the 2EH-exposed group compared to those in the control group but later decreased to the control levels at W4 (Fig. 4B). PCNA-positive cells, markers for proliferating globose basal cells, were mainly observed near BM in the control group but were observed throughout the OE, not only near BM, at D1 in the 2EH-exposed group (Fig. 4C). Number of PCNA-positive cells in OE was significantly lower at W1, W2, and W4 than at D1 and remained lower in the 2EH-exposed group compared to that in the control group (Fig. 4C).

DISCUSSION

During the four-week exposure to 70 ppm 2EH and subsequent recovery period, 2EH induced OE degeneration at W2 and decreased OMP-positive cell proportions at W1 and W2. OE was not immediately repaired, instead the toxic effects reappeared one or two weeks after 2EH cessation. OE was gradually, but not completely, repaired over the four-week recovery period.

To the best of our knowledge, this study is the first to investigate the early time course of OE changes after cessation of inhalation exposure to 2EH. In this study, OE morphological changes were comparable between the control and D1 groups, consistent with our previous report (Miyake *et al.*, 2016) that mice exposed to 60 ppm

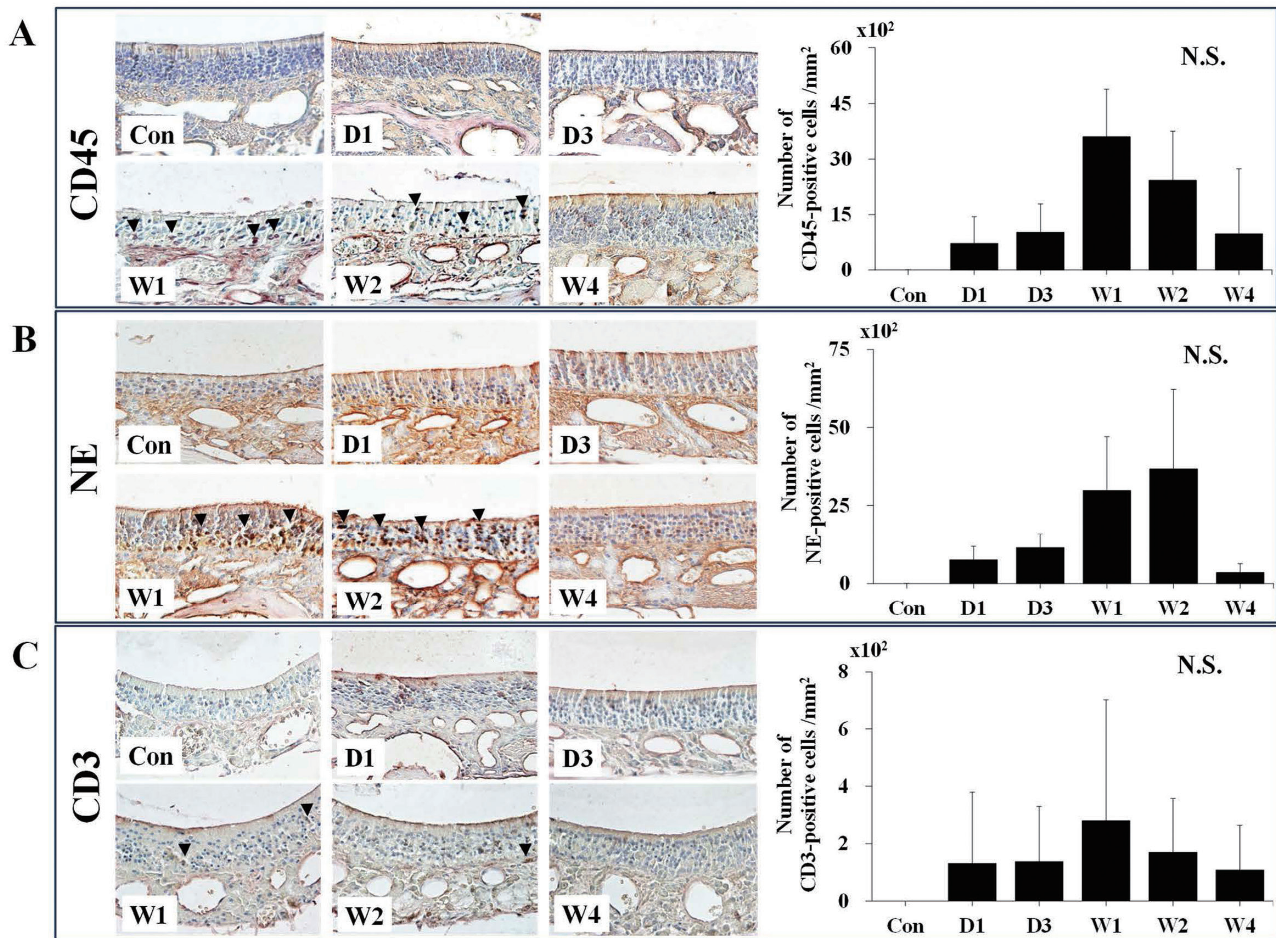

Fig. 3. Immunohistopathological analysis of the infiltrating leukocytes in the OE of mice exposed to 2EH for four weeks, followed by recovery periods of 1 and 3 d and 1, 2, and 4 weeks. Infiltrating leukocytes in mouse OE were immunostained with antibodies against CD45 (A), neutrophil elastase (NE) (B), and CD3 (C). Typical photographs of each group are shown. Arrowheads point to the respective stained cells. Scale bar, 50 µm. The figure on the right shows the number of positive cells (/mm²) for each antibody. Each column and bar represent the mean and SD, respectively (n = 5/group). N.S., not significant.

2EH show acute OE degeneration at one week, followed by OE regeneration at one month despite continuous exposure. Therefore, OE at D1 indicated the OE recovered from the damage at one week.

To date, durations of inhalation exposure and subsequent recovery periods for several substances have been evaluated. Inhalation exposure to ethanol, isobutyraldehyde, and styrene affect the mature olfactory neurons during exposure, which subsequently recover after exposure cessation (Cruzan *et al.*, 2001; Vent *et al.*, 2004). In contrast, effects of 2EH exposure on OMP-positive cells were delayed and observed at W1 and W2. Infiltration of neutrophils and lymphocytes into OE as well as the decreased number of OMP-positive cells damaged the OE tissue and resulted in disorganized cells with numerous gaps in OE. Number of OMP-positive cells decreased shortly after one month, indicating that OMP-positive cells recovered from the damage caused by 2EH at one month, and decreased again three months later in our previous study (Miyake *et al.*, 2016).

Similar to our previous study (Miyake *et al.*, 2016), number of PCNA-positive cells increased, and they were widely distributed throughout OE in the 2EH-exposed group on D1 and lined near BM in the control group. On D1, acute OE degeneration observed within one week of exposure is possibly indicating the ongoing OE repair

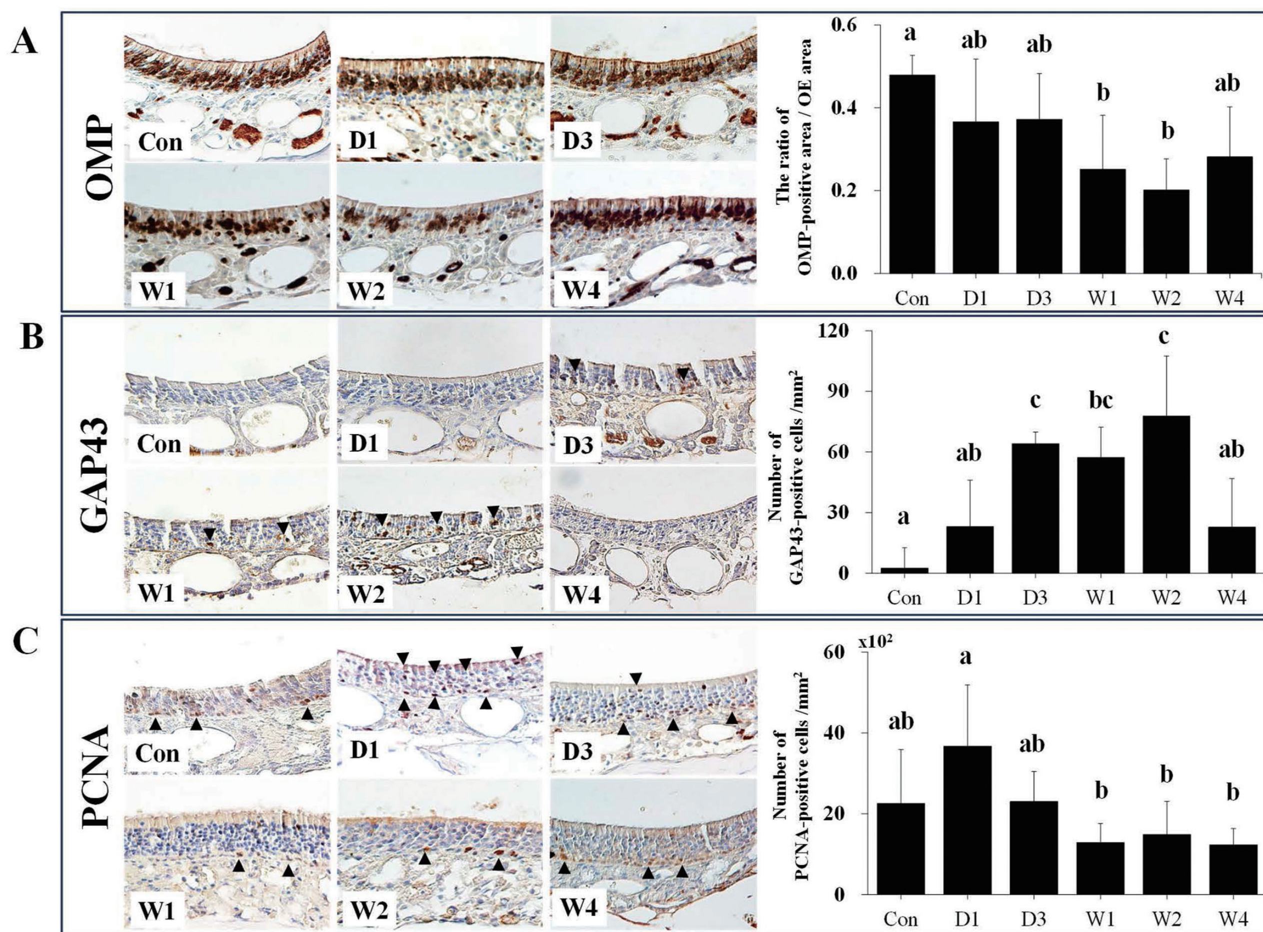

Fig. 4. Expression levels of olfactory nerve-related markers in the OE of mice exposed to 2EH for four weeks, followed by recovery periods of 1 and 3 d and 1, 2, and 4 weeks. Olfactory nerve-related cells in the mouse OE were immunostained with antibodies against the olfactory marker protein (OMP) (A), growth associated protein (GAP)43 (B), and proliferating cell nuclear antigen (PCNA) (C). Typical photographs of each group are shown. Arrowheads point to the respective stained cells. Scale bar, 50 µm. (A) The figure on the right shows the ratio of OMP-positive area to OE area. (B and C) The figure on the right shows the number of positive cells (/mm²) for each antibody. Each column and bar represent the mean and SD, respectively. Different letters above the bars indicate the statistically significant differences ($p < 0.05$; $n = 5$/group).

as PCNA-positive cells are widely distributed when OE was repaired after inhalation exposure of pyridine (Buron *et al.*, 2013). The number of PCNA-positive cells decreased to the control level after D3 and remained low thereafter. As mentioned above, number of OMP-positive cells decreased but the number of PCNA-positive cells did not increase at W1 and W2; the reason for the lack of increase in PCNA-positive cell number remains unknown. Here, increase in GAP43-positive cell number at D3, W1, and W2 corresponded to the decrease in OMP-positive cell number, not relying on the self-proliferation of globose basal cells. As olfactory neurons transition from basal cells to GAP43-positive immature neurons five days after the last cell division of globular basal cells (Schwob *et al.*, 1992), number of GAP43-positive cells reflects the number of PCNA-positive cells several days earlier. Although the number of PCNA-positive cells just before the end of exposure remains unknown, we assumed that it was greater than that in the control group based on the results of continued inhalation exposure for over one month (Miyake *et al.*, 2016). Therefore, it is curious that number of GAP43-positive cells on D1

was not significantly higher than that in the control group. As apoptosis most likely occurs in immature neurons (Mahalik, 1996), survival of GAP43-positive cells is possibly suppressed upon exposure to inhaled 2EH.

A previous study on mice exposed to methylethyleneketoxime via inhalation revealed that exposure to low chemical concentrations for short periods required short periods after exposure for recovery (Newton *et al.*, 2002). This indicates that the recovery time course is not generalizable for the case of exposure to high 2EH concentrations indoor for over one month during hot and humid seasons. Notably, recovery of the olfactory bulb from 2EH exposure could not be determined in this study, but a previous study reported olfactory bulb toxicity even after three months (Miyake *et al.*, 2016).

In conclusion, during the four-week recovery period after 2EH inhalation exposure, OE tissue damage was indicated by the decrease in OMP-positive cell proportions and increase in GAP43-positive cell proportions from D3 to W2, after which the decrease in OMP-positive cell proportions stopped. Although OE was not completely repaired at W4, PCNA-positive cells lined BM, GAP43-positive cell proportions decreased, and OMP-positive cell proportions increased, indicating the potential recovery of OMP-positive cell proportions recovered to control group levels in a few more days.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Aoki, A., Suzaki, H., Kawabata, Y. and Nomura, Y. (1994): Effect of perchloroethylene inhalation on nasal mucosa in mice. Eur. Arch. Otorhinolaryngol., **251**, 361-365.

Bergman, U., Östergren, A., Gustafson, A.L. and Brittebo, B. (2002): Differential effects of olfactory toxicants on olfactory regeneration. Arch. Toxicol., **76**, 104-112.

Buron, G., Hacquemand, R., Pourié, G. and Brand, G. (2009a): Carbon dioxide effects on olfactory functioning: behavioral, histological and immunohistochemical measurements. Toxicol. Lett., **188**, 251-257.

Buron, G., Hacquemand, R., Pourié, G. and Brand, G. (2009b): Inhalation exposure to acetone induces selective damage on olfactory neuroepithelium in mice. Neurotoxicology, **30**, 114-120.

Buron, G., Hacquemand, R., Pourié, G., Jacquot, L. and Brand, G. (2013): Effects of pyridine inhalation exposure on olfactory epithelium in mice. Exp. Toxicol. Pathol., **65**, 159-164.

Chino, S., Kato, S., Seo, J. and Ataka, Y. (2009): Study on emission of decomposed chemicals of esters contained in PVC flooring and adhesive. Build. Environ., **44**, 1337-1342.

Cruzan, G., Cushman, J.R., Andrews, L.S., Granville, G.C., Johnson, K.A., Bevan, C., Hardy, C.J., Coombs, D.W., Mullins, P.A. and Brown, W.R. (2001): Chronic toxicity/oncogenicity study of styrene in CD-1 mice by inhalation exposure for 104 weeks. J. Appl. Toxicol., **21**, 185-198.

Harkema, J.R., Carey, S.A. and Wagner, J.G. (2006): The nose revisited: a brief review of the comparative structure, function, and toxicologic pathology of the nasal epithelium. Toxicol. Pathol., **34**, 252-269.

Hext, P.M., Pinto, P.J. and Gaskell, B.A. (2001): Methyl methacrylate toxicity in rat nasal epithelium: investigation of the time course of lesion development and recovery from short term vapour inhalation. Toxicology, **156**, 119-128.

Jacquot, L., Pourie, G., Buron, G., Monnin, J. and Brand, G. (2006): Effects of toluene inhalation exposure on olfactory functioning: behavioral and histological assessment. Toxicol. Lett., **165**, 57-65.

Jang, W., Youngentob, S.L. and Schwob, J.E. (2003): Globose basal cells are required for reconstitution of olfactory epithelium after methyl bromide lesion. J. Comp. Neurol., **460**, 123-140.

Kamijima, M., Sakai, K., Shibata, E., Yamada, T., Itohara, S., Ohno, H., Hayakawa, R., Sugiura, M., Yamaki, K. and Takeuchi, Y. (2002): 2-Ethyl-1-hexanol in indoor air as a possible cause of sick building symptoms. J. Occup. Health, **44**, 186-191.

Kamijima, M., Shibata, E., Sakai, K., Ohno, H., Ishihara, S., Yamada, T., Takeuchi, Y. and Nakajima, T. (2005): [Indoor air pollution due to 2-ethyl-1-hexanol airborne concentrations, emission sources and subjective symptoms in classroom users]. Nihon Koshu Eisei Zasshi, **52**, 1021-1031.

Mahalik, T.J. (1996): Apparent apoptotic cell death in the olfactory epithelium of adult rodents: death occurs at different developmental stages. J. Comp. Neurol., **372**, 457-464.

Menco, B.P. (1989): Electron-microscopic demonstration of olfactory-marker protein with protein G-gold in freeze-substituted, Lowicryl K11M-embedded rat olfactory-receptor cells. Cell Tissue Res., **256**, 275-281.

Miyake, M., Ito, Y., Sawada, M., Sakai, K., Suzuki, H., Sakamoto, T., Sawamoto, K. and Kamijima, M. (2016): Subchronic inhalation exposure to 2-ethyl-1-hexanol impairs the mouse olfactory bulb via injury and subsequent repair of the nasal olfactory epithelium. Arch. Toxicol., **90**, 1949-1958.

Newton, P.E., Bolte, H.F., Derelanko, M.J., Hardisty, J.F. and Rinehart, W.E. (2002): An evaluation of changes and recovery in the olfactory epithelium in mice after inhalation exposure to methylethylketoxime. Inhal. Toxicol., **14**, 1249-1260.

Sakai, K., Kamijima, M., Shibata, E., Ohno, H. and Nakajima, T. (2009): Annual transition and seasonal variation of indoor air pollution levels of 2-ethyl-1-hexanol in large-scale buildings in Nagoya, Japan. J. Environ. Monit., **11**, 2068-2076.

Scala, R.A. and Burtis, E.G. (1973): Acute toxicity of a homologous series of branched-chain primary alcohols. Am. Ind. Hyg. Assoc. J., **34**, 493-499.

Schwob, J.E., Szumowski, K.E. and Stasky, A.A. (1992): Olfactory sensory neurons are trophically dependent on the olfactory bulb for their prolonged survival. J. Neurosci., **12**, 3896-3919.

Vent, J., Robinson, A.M., Gentry-Nielsen, M.J., Conley, D.B., Hallworth, R., Leopold, D.A. and Kern, R.C. (2004): Pathology of the olfactory epithelium: smoking and ethanol exposure.

Laryngoscope, **114**, 1383-1388.
Wakayama, T., Ito, Y., Sakai, K., Miyake, M., Shibata, E., Ohno, H. and Kamijima, M. (2019a): Comprehensive review of 2-ethyl-1-hexanol as an indoor air pollutant. J. Occup. Health, **61**, 19-35.
Wakayama, T., Sakai, K., Ohno, H., Ito, Y. and Kamijima, M. (2019b): Reexamination in a building with high concentration of 2-ethyl-1-hexanol. In: The 89th Annual Meeting of the Japanese Society for Hygiene, pp. 169, Nagoya.
Wieslander, G., Norbäck, D., Nordström, K., Wålinder, R. and Venge, P. (1999): Nasal and ocular symptoms, tear film stability and biomarkers in nasal lavage, in relation to building-dampness and building design in hospitals. Int. Arch. Occup. Environ. Health, **72**, 451-461.