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**ORIGINAL ARTICLE**

# Assessment of the time-dependent dermatotoxicity of mechlorethamine using the mouse ear vesicant model

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## ABSTRACT

Mechlorethamine (HN2) is an alkylating agent and sulfur mustard gas mimetic which is also used in anticancer therapy. HN2 is associated with skin inflammation and blistering which can lead to secondary infections. The purpose of the present study was to investigate the time-dependent dermatotoxicity of HN2 using the mouse ear vesicant model (MEVM). To this end, our operational definition of dermatotoxicity included tissue responses to HN2 consistent with an increase in the wet weights of mouse ear punch biopsies, an increase in the morphometric thickness of H&E stained ear sections and histopathologic observations including tissue edema, inflammatory cell infiltration and vesication. The ears of male Swiss Webster mice were topically exposed to a single dose of HN2 (0.5 µmol/ear) or DMSO vehicle (5 µl/ear) or left untreated (naive). Mice were then euthanized at 15 min, 1, 2, 4, 8 or 24 hr following HN2 exposure. Compared to control ears, mouse ears exposed to HN2 at all time points showed an increase in wet weights, morphometric thickness, edema, inflammatory cell infiltration and signs of vesication. The incidence in tissue vesication sharply increased between 4 and 8 hr after exposure, revealing that tissue vesication is well established by 8 hr and remains elevated at 24 hr after exposure. It is noteworthy that, compared to control ears, mouse ears treated with DMSO vehicle alone also exhibited an increase in wet weights and morphometric thickness at 15 min, 1, 2 and 4 hr following treatment; however, these vehicle effects begin to subside after 4 hr. The results obtained here using the MEVM provide a more holistic understanding of the kinetics of vesication, and indicate that time points earlier than 24 hr may prove useful not only for investigating the complex mechanisms involved in vesication but also for assessing the effects of vesicant countermeasures.

**KEY WORDS:** mechlorethamine; mouse ear vesicant model; MMP-9; dermatotoxicity; vesication

## Introduction

Mechlorethamine (HN2) is a prototype nitrogen mustard (NM) that shares a similar structure and toxicity profile with the chemical warfare agent sulfur mustard (SM) (Korkmaz *et al.*, 2006; Shakarjian *et al.*, 2010). HN2 is widely used as a surrogate to study and mimic the effects of SM under laboratory conditions (Composto *et al.*, 2018; Lulla *et al.*, 2014; Malaviya *et al.*, 2015; Sunil *et al.*, 2011; Tumu *et al.*, 2018).

Due to the presence of the highly reactive chloroethyl side chains in both compounds, both SM and NM readily interact with a wide variety of macromolecules including proteins and nucleic acids. Upon absorption into the aqueous components of the body, SM and NM transform into the reactive intermediates ethylene sulfonium and ethylene immonium ions, respectively. These reactive intermediates cause direct injury to tissues through their interaction with proteins and DNA, particularly through their ability to form both bifunctional DNA interstrand cross links and monofunctional DNA adducts which can subsequently block DNA replication and lead to cell cycle arrest (Kehe *et al.*, 2009). Moreover, HN2 and SM have been reported to alkylate cytoskeletal proteins such as keratin 5 and keratin 14 in epidermal keratinocytes and to affect proteins in the extracellular matrix such as laminin-332 (Shakarjian *et al.*, 2010). Laminin-332 is regarded as a supramolecular bridge between the basal keratinocytes of the epidermis and the underlying dermis (Kiritsi *et al.*, 2013). In addition, sulfhydryl groups, such as those found in key cellular enzymes or in reduced glutathione (GSH) are a common site for protein and peptide

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alkylation by mustards (Kehe *et al.,* 2009; Korkmaz *et al.,* 2006; Shakarjian *et al.,* 2010).

Part of the toxicity of SM and HN2 may stem from depletion of cellular glutathione stores which, in turn, leads to the intracellular accumulation of reactive oxygen species (ROS) and oxidative DNA damage (Crater and Kannan, 2007; Pant and Lomash, 2016; Paromov *et al.,* 2007). The combination of oxidative DNA damage and direct DNA alkylation leads to strand breaks and activates polymerases such as poly(adenosine diphosphate-ribose) polymerase (PARP). When PARPs react with cellular proteins following exposure to SM, a marked depletion in nicotinamide adenine dinucleotide (NAD+) and adenosine triphosphate (ATP) has been observed. The depletion of NAD+ is associated with reduced glucose consumption and lactate formation, whereas low ATP levels contribute to necrotic cell death (Kehe *et al.,* 2009). Thus, following exposure to SM and subsequent overactivation of PARP, ATP is depleted and degradative enzymes including matrix metalloproteinases (MMPs) and serine proteases are released and thus contributing to tissue destruction (Kehe *et al.,* 2009).

The vesicant actions of SM or HN2 are likely due, at least in part, to the upregulation of MMPs capable of degrading extracellular macromolecules and contributing to epidermal:dermal detachments (Shakarjian *et al.,* 2010). To this end, topically applied SM led to the expression of MMP-9 in a 3D-skin model (Ries *et al.,* 2009), while mouse ears topically exposed to SM (Shakarjian *et al.,* 2006) or HN2 (Tumu *et al.,* 2018) exhibited an increase in MMP-9 expression relative to control tissues MMP-9, also increased in mustard-exposed rat lungs (Sunil *et al.,* 2011; Malaviya, *et al.,* 2010) and mustard-exposed corneas (Gordon *et al.,* 2016).

Despite several decades of research, a highly efficacious countermeasure to SM toxicity in humans has yet to be developed. Moreover, the process of tissue vesication, also known as epidermal:dermal detachment remains to be fully characterized. The main purpose of this study was to use the SM surrogate HN2 to better understand the process of tissue vesication and to investigate the approximate time when subepidermal blister formation occurs after HN2 exposure. This knowledge is necessary for the development of antidotes aimed at reducing the vesicant activity of HN2 . The mouse ear vesicant model (MEVM) was used here to investigate the time-dependent dermatotoxicity of HN2 *in vivo.* The dermatotoxic endpoints investigated included tissue edema, as determined by measurement of tissue wet weights and thickness, tissue expression of MMP-9, as determined by immunohistochemistry (IHC), and vesication, as determined from light microscopy of H&E stained tissue sections.

## Materials and methods

### Chemicals, reagents, and other materials

Mechlorethamine hydrochloride (HN2) was purchased from Pfaltz & Bauer (Waterbury, CT; Cat # 55-86-7). Dimethyl sulfoxide (DMSO) was obtained from J.T. Baker (Philipsburg, NJ; Cat# 67-68-5). A dose of 0.5 µmol/ear HN2 was used in the present study. DMSO was used as the vehicle for HN2 due to its ability to readily penetrate the skin.

Eosin (Cat # CA95057-848), hematoxylin (Cat # CA95057-844), xylene (Cat # CA95057-822), histology grade 100% ethanol (Cat # CA95057-828) and Paraplast X-tra (Cat # 15159-486 -1 kg) were purchased from VWR International (West Chester, PA). Buffered formalin (1:10 dilution, already diluted) (Cat # 23-245-685) was purchased from Fisher Scientific (Nazareth, PA). Permount was purchased from Fisher Scientific (Fairlawn, NJ; Cat# SP15-500). Isoflurane (Cat # 029405) was purchased from Henry Schein (Dublin, OH). Slides and cover glasses were also purchased from VWR International (Radnor, PA; Cat# 16004-386 and Cat # 48382-136, respectively). Vectastain ABC Rabbit IgG Kit (Cat # PK-6101) and Antigen Unmasking Solution (Citrate Based) (Cat # H-3300) were both purchased from Vector Laboratories (Burlingame, CA). Phosphate buffered saline (PBS) (10X) liquid concentrate was obtained from EMD Millipore (Gibbstown, NJ; Cat # 6505-OP). Tris Buffered Saline (TBS) (10X) was purchased from VWR International (West Chester, PA; Cat # 10128-548). The 100% n-butanol was purchased from EMD Millipore (Billerica, MA; Cat # BX1777-6). Tween-20 was purchased from VWR International (Solon, OH; Cat # 97062-332). 30% Hydrogen Peroxide ($H_2O_2$) was purchased from VWR International (Mississauga, ON, Cat # BDH7690-1).

### Animal studies

The protocol for this research was approved by the Institutional Animal Care and Use Committee (IACUC) of St. John's University and the animals were cared for in accordance with the guidelines established by the U.S. Department of Agriculture (USDA). Outbred male Swiss Webster mice were purchased from Taconic farms (Germantown, NY). All mice were kept and maintained in the AAALAC-accredited Animal Care Center at St. John's University (Queens, NY). Animals were allowed to adjust to the new environment for at least 2–3 days before use. All animals were housed in groups of 2–4 per cage in temperature and humidity regulated rooms with 12 hour day and 12 hour night cycles. The total number of mice that were used for the HN2 time course study was 72.

The MEVM has been utilized previously to investigate various histopathological parameters of skin exposed to mustards (Brinkley *et al.,* 1989; Casillas *et al.,* 1997, 2000; Dachir *et al.,* 2002). Common skin responses that are evaluated using the MEVM include edema, hyperplasia, dermal:epidermal separation (vesication), inflammatory cell infiltration and epidermal necrosis. In particular, the strength of the MEVM is that allows for a quantitative measure of vesication.

### Test solutions and reagents

(a) HN2 solution: mechlorethamine hydrochloride in the amount of 0.0192 g (molecular weight: 192.52) was

dissolved in 1 ml of DMSO to obtain a 0.100 M of HN2. When 5 µl of this solution is applied to the inner surface of a mouse ear, it is equal to a HN2 dose of 0.5 µmol/ear. This dose has previously been used by our lab to induce the formation of subepidermal microblisters in Swiss Webster mice (Lulla *et al.,* 2014; Tumu *et al.,* 2018).

(b) Other reagents. *Vehicle*: a 5 µl volume of DMSO was applied to the ears that served as controls in vehicle control tissues. *Buffered formalin solution (1:10 dilution):* Neutral buffered formalin (8 ml) was used for fixation of each ear punch. *Dehydration alcohol solutions:* Histology grade dehydration ethanol 100% was diluted with distilled and deionized water to obtain 30%, 60%, 70% and 95% ethanol concentrations. *Hematoxylin staining solution*: Ready-made hematoxylin (H) solution purchased from VWR was used to stain the nuclei of the tissue sections. *Eosin staining solution*: Ready-made eosin (E) solution purchased from VWR was used to stain the cytoplasm of the tissue sections.

#### HN2 time course study

Wild type, male Swiss Webster mice weighing between 25–30 g were separated into different groups. For HN2 treated mice, the right ears were treated with a 5 µl volume of 0.100 M HN2, while the left ears served as test controls and received a 5 µl volume of DMSO. For mice treated with DMSO only, the right ears were treated with a 5 µl volume of DMSO, while the left ears were left untreated (naive). Next, at 15 min, 1 hr, 2 hr, 4 hr, 8 hr or 24 hr after exposure to HN2 or DMSO only, blood was collected from anesthetized mice via close chested cardiac puncture. Animals were then euthanized in a carbon dioxide ($CO_2$) chamber and ear tissue samples were collected using an 8 mm biopsy punch and weighed on an analytical balance. The ear samples were then transferred to 20 ml vials with 8–10 ml neutral buffered formalin for 18–24 hr before dehydration and embedding in paraffin. Tissue sections of 5 µm thickness were sectioned using a standard rotary microtome, placed in a water bath at 40 °C that also contained few drops of 5% gelatin. Sections were lifted from the water onto the slides and dried overnight. The next day, slides without visible tissue section tears were selected for staining with H & E using a standard staining protocol.

#### Morphometric analysis

Ear thickness was measured using the camera of a Motic BA210 microscope and Motic Image plus 2.0 software. The H & E stained sections were measured under a total magnification of 40× with software calibrated measurements. Thickness of the ear tissues was measured by drawing several perpendicular lines from one side of the tissue to the other side. Nine such lines were drawn on each section and equally spaced along the section and the average distance from one epidermis to the other epidermis was determined. Two duplicate slides for each ear were used in the morphometric analysis. In total, the average of eighteen measurements was taken for each ear sample.

#### Histopathological evaluation

A Zeiss Axio Scope A1 microscope equipped with Axiocam 506 color camera and Zeiss Zen 2.3 software was used for obtaining light microscopy images of immunohistochemistry (IHC) and H & E stained tissues. Stained tissues were evaluated for vesication (epithelial detachment) using a "+" or "–" system by three blinded investigators. Each blinded investigator was instructed to assign a positive score for vesication (+) if the tissue section was found to possess at least one epidermal:dermal detachment. It should be noted that two different tissue sections from each ear punch were scored by each of the three blinded scorers, leading to a total of six scores per ear sample. Lastly, an ear sample was counted as "+" for vesication when at least four of the combined six scores for a particular ear sample were in agreement.

#### IHC for MMP-9

IHC for MMP-9 was carried out as described previously (Tumu *et al.,* 2018).

#### Statistical analysis

All results are presented as the mean ± SEM for 6 or 12 ear samples, depending on the experiment. Statistical significance for wet weight and morphometric analysis was tested between groups using a one-way ANOVA followed by Newman-Keuls multiple comparison post hoc analysis and GraphPad Prism® version 5.0 software.

## Results

For each time point studied there were two groups of mice (N=6 per group). In the first group, mouse ears were treated with HN2 (0.5 µmol/ear) on the right ear and DMSO on the left ear. In the second group, mouse ears were treated with DMSO on the right ear, with the left ear untreated (naive). Mice were euthanized at 15 min, 1 hr, 2 hr, 4 hr, 8 hr or 24 hr following exposure to HN2 or DMSO.

Tissue biopsy wet weights were used as a rudimentary indicator of edema. It is noteworthy that when ear tissues treated with DMSO vehicle alone were compared to untreated naive ear tissues, significant increases in tissue biopsy wet weights were observed at 15 min (1.47 fold vs. naïve), 1 hr (1.84 fold vs. naïve) and 2 hr (1.78 fold vs. naïve) following topical application, as well as at 4 hr (1.46 fold vs. naïve), but not at 8 hr (1.14 fold vs. naïve) or 24 hr (1.01 fold vs. naïve) (Figure 1). When ear punch biopsies were obtained from mice treated with HN2 and compared to those obtained from mice treated with DMSO alone, significant increases in tissue wet weights were observed at 15 min (1.19 fold vs. DMSO), 1 hr (1.17 fold vs. DMSO), 2 hr (1.53 fold vs. DMSO), 4 hr (2.01 fold vs. DMSO), 8 hr (3.05 fold vs. DMSO) and 24 hr (2.84 fold vs. DMSO) (Figure 1). Thus, ear swelling was observed to be time-dependent, with maximum swelling observed 8 hr after topical application of HN2.

Morphometric thickness of ear tissues was then assessed using light microscopy. Compared to tissue

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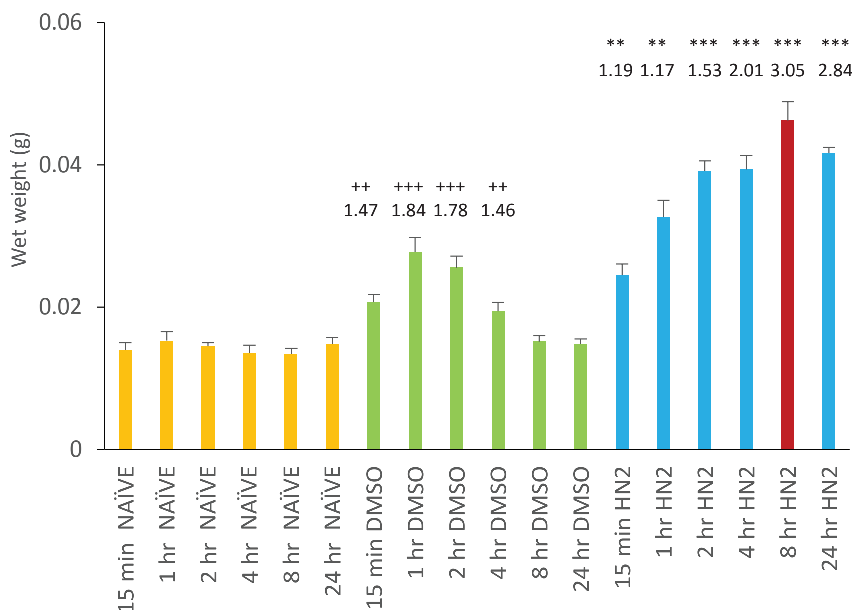


**Figure 1.** Wet weight analysis of ear punches obtained from male Swiss Webster mice. Ears were treated with either HN2 (0.5 µmol/ear) or DMSO or left untreated (naïve) and euthanized at 15 min, 1 hr, 2 hr, 4 hr, 8 hr or 24 h following topical exposure. Statistical differences were observed between naïve tissues and DMSO only treated ears at 15 min and 4 hr after treatment which were significant at ++$p$<0.01, whereas DMSO only treated ears were significantly different from naïve tissues at 1 hr or 2 hr at +++$p$<0.001. Note that fold changes relative to naïve are indicated above each bar. Statistical differences between HN2 and DMSO groups at 15 min and 1 hr were significant at **p<0.01, whereas HN2 treated ears were significantly different from DMSO treated ears 2 hr, 4 hr, 8 hr and 24 hr at ***$p$<0.001. Note that fold changes relative to DMSO only are indicated above each bar. Data represent average weights ± SEM.

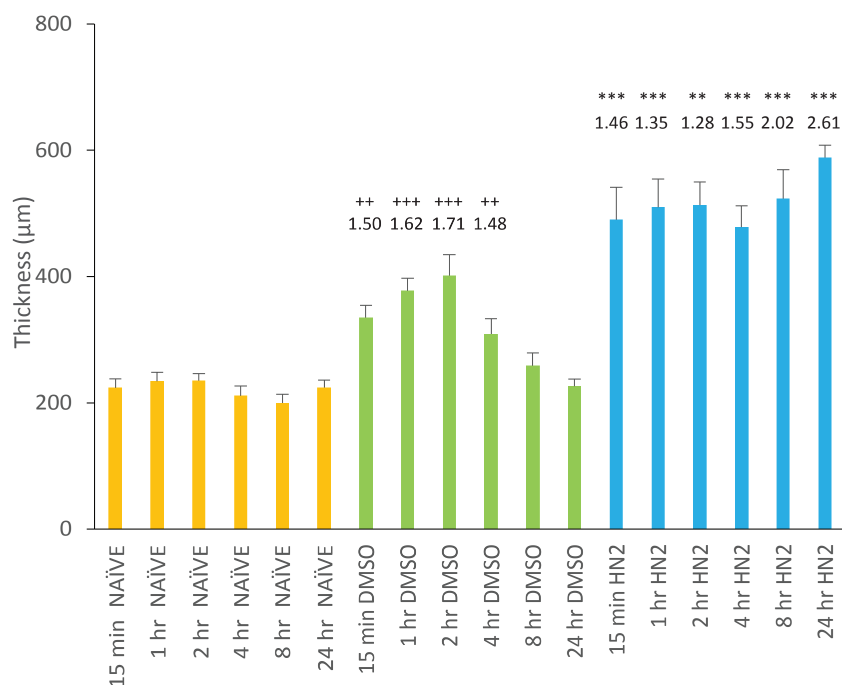


**Figure 2**. Morphometric analysis of ear punches obtained from male Swiss Webster mice. Ears were treated with either HN2 (0.5 µmol/ear) or DMSO or left untreated (naïve) and euthanized at 15 min, 1 hr, 2 hr, 4 hr, 8 hr or 24 h following topical exposure. Statistical differences were observed between naïve tissues and DMSO only treated ears 15 min and 4 hr after treatment which were significant at ++$p$<0.01, whereas DMSO only treated ears were significantly different from naïve tissues at 1 and 2 hr after treatment at +++$p$<0.001. Note that fold changes relative to naïve are indicated above each bar. Statistical differences between HN2 treated ear tissues and DMSO only treated ears ear tissues were significant at 15 min, 1 hr and 2 hr at **$p$<0.01 and at 4 hr, 8 hr and 24 hr following HN2 exposure at ***$p$<0.001. Note that fold changes relative to DMSO only are indicated above each bar. Data represent average weights ± SEM.

sections from naïve ears, those treated with DMSO vehicle alone showed a significant increase in morphometric thickness at 15 min (1.50 vs. naïve), 1 hr (1.62 vs. naïve), 2 hr (1.71 fold vs. naïve) and 4 hr (1.48 fold vs. naïve) but not at 8 hr (1.30 fold vs. naïve, p=0.052) or 24 hr (1.00 fold vs. naïve) (Figure 2). When ear punch biopsies were obtained from mice treated with HN2 and compared to ear punch biopsies obtained from mice treated with DMSO alone, significant increases in morphometric thickness were observed at 15 min (1.46 fold vs. DMSO), 1 hr (1.35 fold vs. DMSO), 2 hr (1.28 fold vs. DMSO), 4 hr (1.55 fold vs. DMSO), 8 hr (2.02 fold vs. DMSO) and 24 hr (2.61 fold vs. DMSO) (Figure 2). Morphometric thickness analysis therefore revealed that ear swelling was time-dependent, occurring in as little as 15 min with maximum tissue thickness observed 24 hr after topical application of HN2.

Histopathologic assessment of H&E stained ear tissues demonstrated that untreated naive ears exhibited two epidermal cell layers, representing the external and internal surfaces of the ear, both approximately one cell in thickness; moreover, these sections showed normal cartilage and sebaceous glands (Figure 3, Panels A and B; Figure 4, Panels A and B). Tissues that were treated with HN2 and then harvested after 15 min (Figure 3, Panel D), 1 hr (Figure 3, Panel F), 2 hr (Figure 3, Panel H), 4 hr (Figure 4, Panel D), 8 hr (Figure 4, Panel F) or 24 hr (Figure 4, Panel H) showed a time-dependent increase in tissue edema and infiltrating immune cells, with minor tissue injury evident in as little as 15 min after HN2 exposure and severe edema and inflammatory cell infiltrates observed at time points ≥1 hr. Maximum injury, including the presence of epidermal:dermal detachments, was observed at time points ≥8 hr. Note that the present study did not examine tissue responses beyond 24 hr.

Compared to naïve ears, ear tissues treated with DMSO alone and collected 15 min after exposure showed a slight increase in tissue swelling (Figure 3, Panel C). The effects of DMSO after topical application to mouse ear skin became more pronounced at 1 hr (Figure 3, Panel E), 2 hr (Figure 3, Panel G) and 4 hr (Figure 4, Panel C), with tissue sections showing edema and increased inflammatory cells at these time points. The worst injury caused by exposure to DMSO alone was observed 2 hr after topical application (Figure 3, Panel G). Ear samples treated topically with DMSO and collected after 8 hr (Figure 4, Panel E) or 24 hr (Figure 4, Panel G) looked similar to naïve ear tissues (Figure 4, Panels A and B). Thus the effects of DMSO alone on tissue edema were transient and subsided by 8 hr after treatment. Moreover, ears treated with HN2 showed more severe tissue injury at every time point than ears treated with DMSO alone. All in all, the light micrographs supported the data obtained from wet weight and morphometric tissue thickness analyses (see Figures 1 and 2).

To investigate the extent to which DMSO or HN2 affects tissue expression of MMP-9, IHC was performed on ear punch biopsies collected 15 min, 1, 2, 4, 8 or 24 hr after topical application (Figure 5 and Figure 6). Whereas naïve ear tissues showed very low expression of MMP-9 (Figure 5, Panels A and B; Figure 6, Panels A and B), ear tissues treated topically with HN2 showed a time-dependent increase in its expression. To this end, MMP-9 was initially observed near the HN2 exposed side of the dermis at 15 min (Figure 5, Panel D) and 1 hr (Figure 5, Panel F) after exposure, but then found to be expressed throughout the tissue and specifically within infiltrating inflammatory cells at 2 hr (Figure 5, Panel H) and 4 hr (Figure 6, Panel D) following HN2 exposure. MMP-9 staining remained elevated throughout the tissue at 8 hr (Figure 6, Panel F) and 24 hr (Figure 6, Panel H) after HN2 exposure, with expression observed not only within inflammatory cells but also in extracellular regions.

Treatment of mouse ears with DMSO alone had an unexpected transient effect on MMP-9 expression which was not observed to persist beyond 4 hr. To this end, when compared to naïve ear tissues (Figure 5, Panels A and B) or ear tissues exposed to DMSO alone and collected 15 min later (Figure 5, Panel C), ear samples obtained at 1 hr after topical application of DMSO exhibited slightly higher expression of MMP-9 within the dermis (Figure 5, Panel E). By 2 hr after DMSO exposure, ear sections showed abundant expression of MMP-9 in both the epidermis and the infiltrating immune cells (Figure 5, Panel G) which persisted until 4 hr after DMSO exposure (Figure 6, Panel C). Ear samples treated topically with DMSO and collected after 8 hr (Figure 6, Panel E) or 24 hr (Figure 6, Panel G) looked similar to naïve ear tissues.

All in all, the tissue expression of MMP-9 in ears treated with HN2 (0.5 µmol/ear) at 4, 8 and 24 hr after topical application (Figure 6, Panels D, F and H, respectively) was dramatically increased compared to tissue expression observed at earlier time points including 15 min, 1 hr and 2 hr (Figure 5, Panels D, F and H, respectively). A transient increase in tissue expression of MMP-9 was observed after topical exposure to DMSO at early time points (1 hr, 2 hr and 4 hr) which was not observed at time points ≥8 hr.

Inspection of H&E sections prepared from ear tissues exposed to HN2 and harvested after 15 min, 2 hr or 4 hr revealed vesication in 16.7% of ears. Ears treated with HN2 and collected after 1 hr did not show any signs of vesication. Ears exposed to HN2 and harvested 8 hr later exhibited vesication in 66.7% of the samples. Ears exposed to HN2 and collected 24 hr later showed vesication in 83.3% of the ears (Table 1; Figure 4, Panel H). Ears exposed to DMSO alone did not show vesication.

## Discussion

Previous studies using the MEVM to investigate mustard toxicity have examined ear tissues for signs of injury at times points such as 6, 12, 18 or 24 hr after exposure (Casillas *et al.*, 1997; Dachir *et al.*, 2002; Shakarjian *et al.*, 2006; Tumu *et al.*, 2018), or have included even later time points such as 72, 120 or 168 h after exposure (Dachir *et al.*, 2002; Shakarjian *et al.*, 2006; Tewari-Singh *et al.*, 2013). In the present study, we investigated the effects of HN2 on the ear skin of male Swiss Webster mice at

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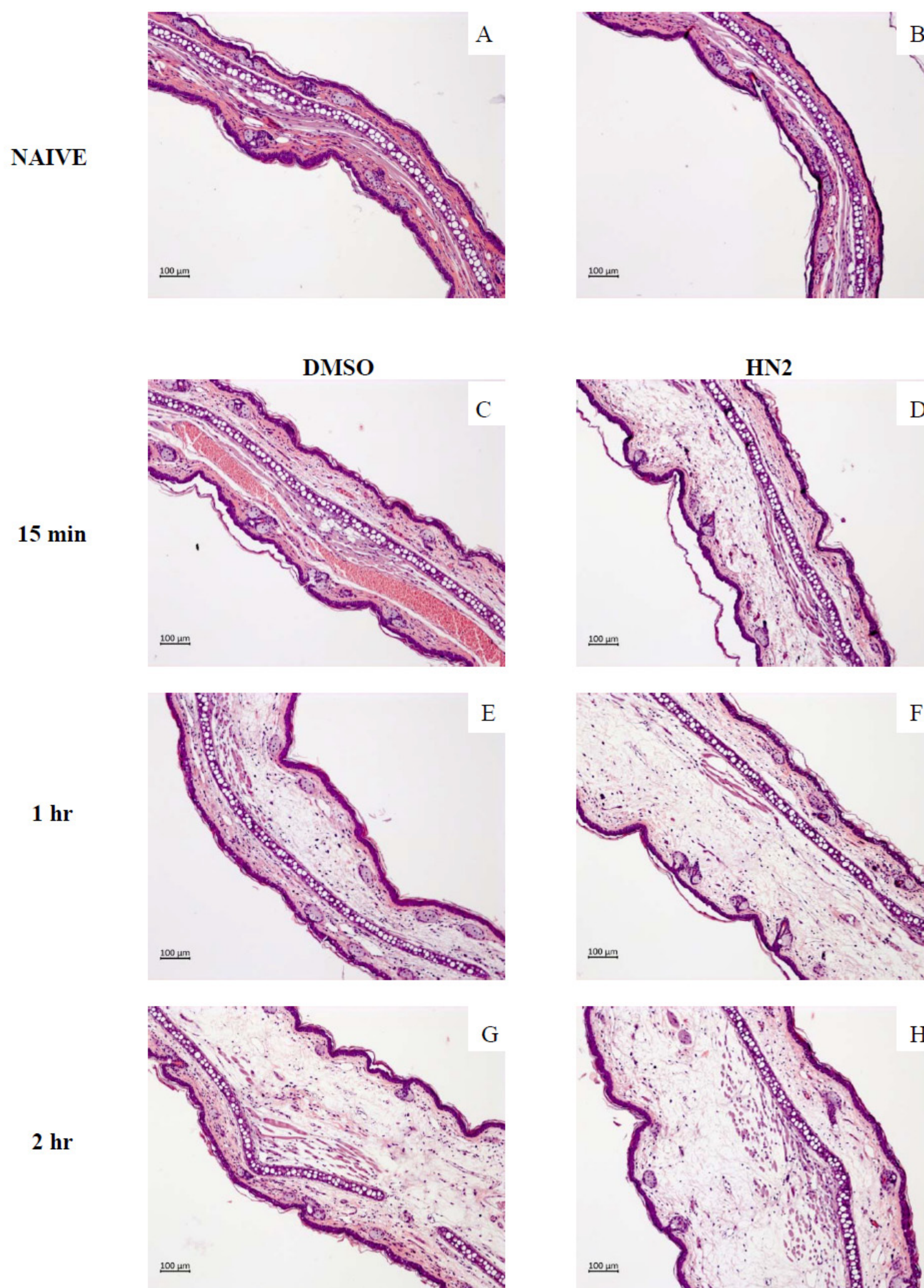


**Figure 3.** Representative light micrographs of H & E stained ear tissue sections obtained from male Swiss Webster mice. Panels A and B represent untreated (naïve) ear tissues. Panels C, E and G represent ears treated with the vehicle DMSO only, and Panels D, F and H represent ears treated with HN2 (0.5 µmol/ear) at 15 min, 1 hr and 2 hr, respectively. Total magnification used is 100X. Scale bars on the micrographs represent a length of 100 µm.

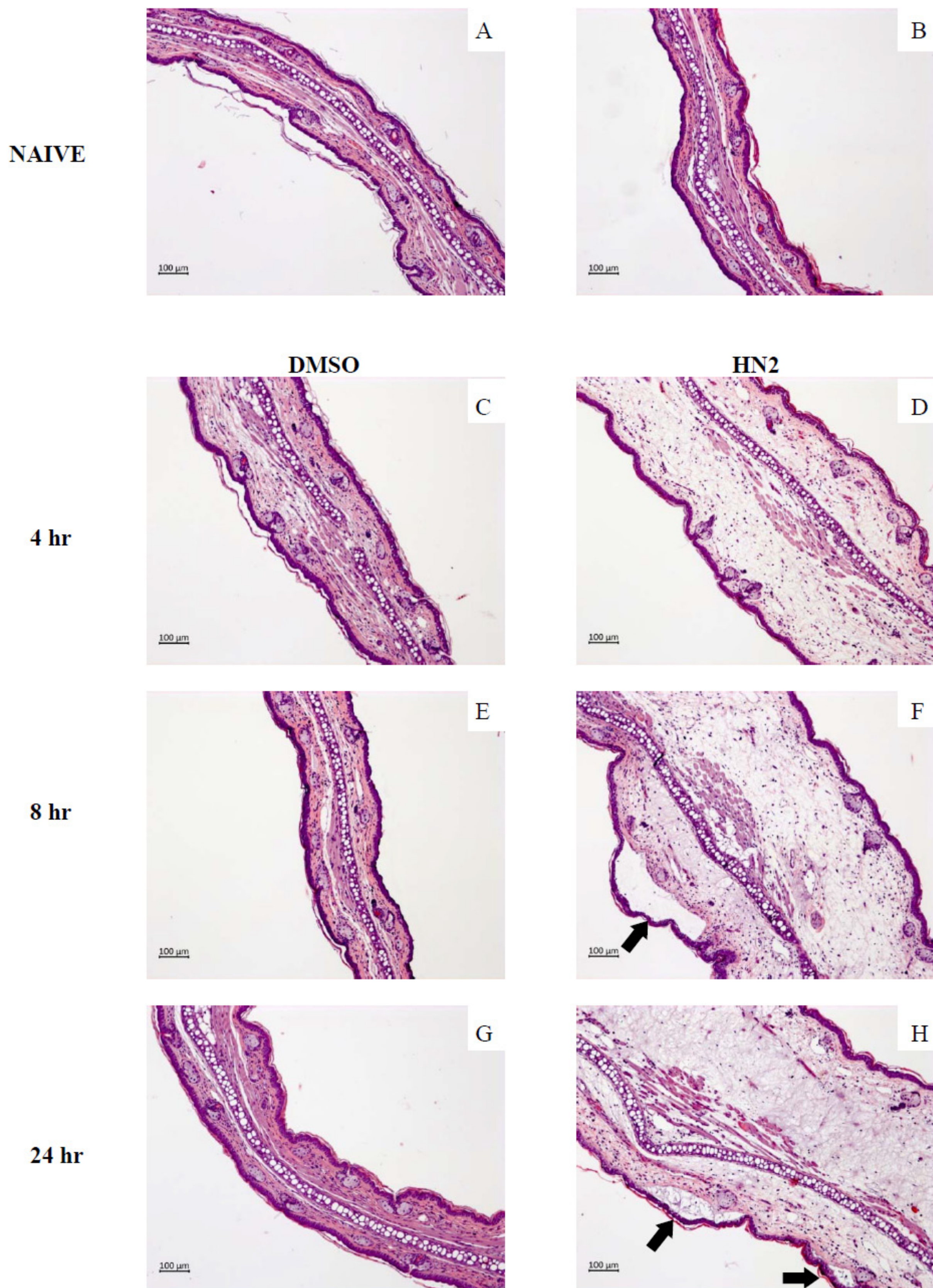


**Figure 4.** Representative light micrographs of H & E stained ear tissue sections obtained from male Swiss Webster mice. Panels A and B represent untreated (naïve) ear tissues. Panels C, E and G represent ears treated with the vehicle DMSO only, and Panels D, F and H represent ears treated with HN2 (0.5 µmol/ear) at 4 hr, 8 hr and 24 hr, respectively. Note the presence of epidermal:dermal detachments in Panels F and H as indicated by the black arrows. Total magnification used is 100X. Scale bars on the micrographs represent a length of 100 µm.

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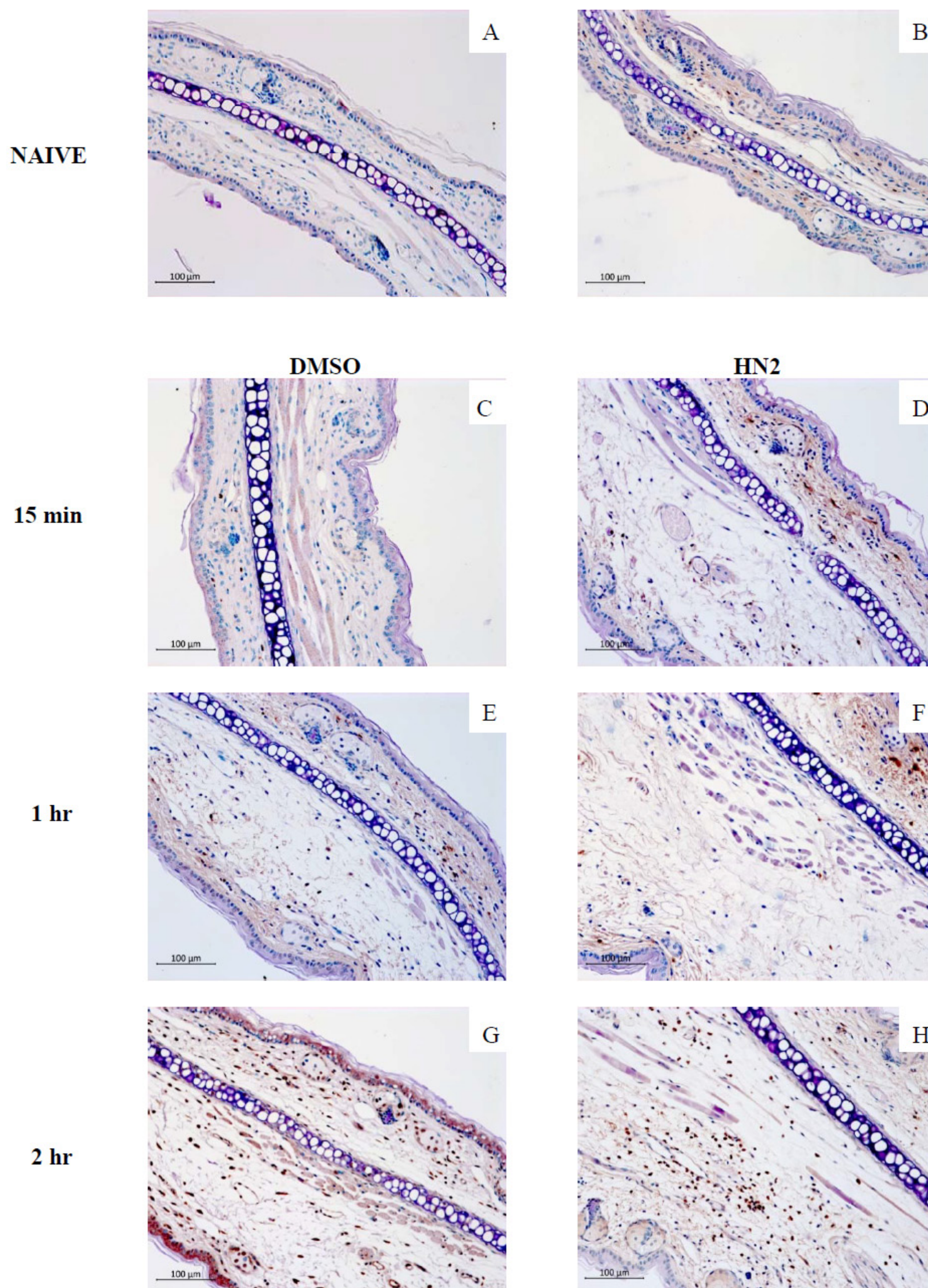


**Figure 5.** Representative light micrographs of IHC staining for MMP-9 in ear tissue sections obtained from male Swiss Webster mice. Panels A and B represent untreated (naïve) ear tissues. Panels C, E and G represent ears treated with the vehicle DMSO only, and Panels D, F and H represent ears treated with HN2 (0.5 µmol/ear) at 15 min, 1 hr and 2 hr, respectively. Total magnification used is 200×. Scale bars on the micrographs represent a length of 100 µm.

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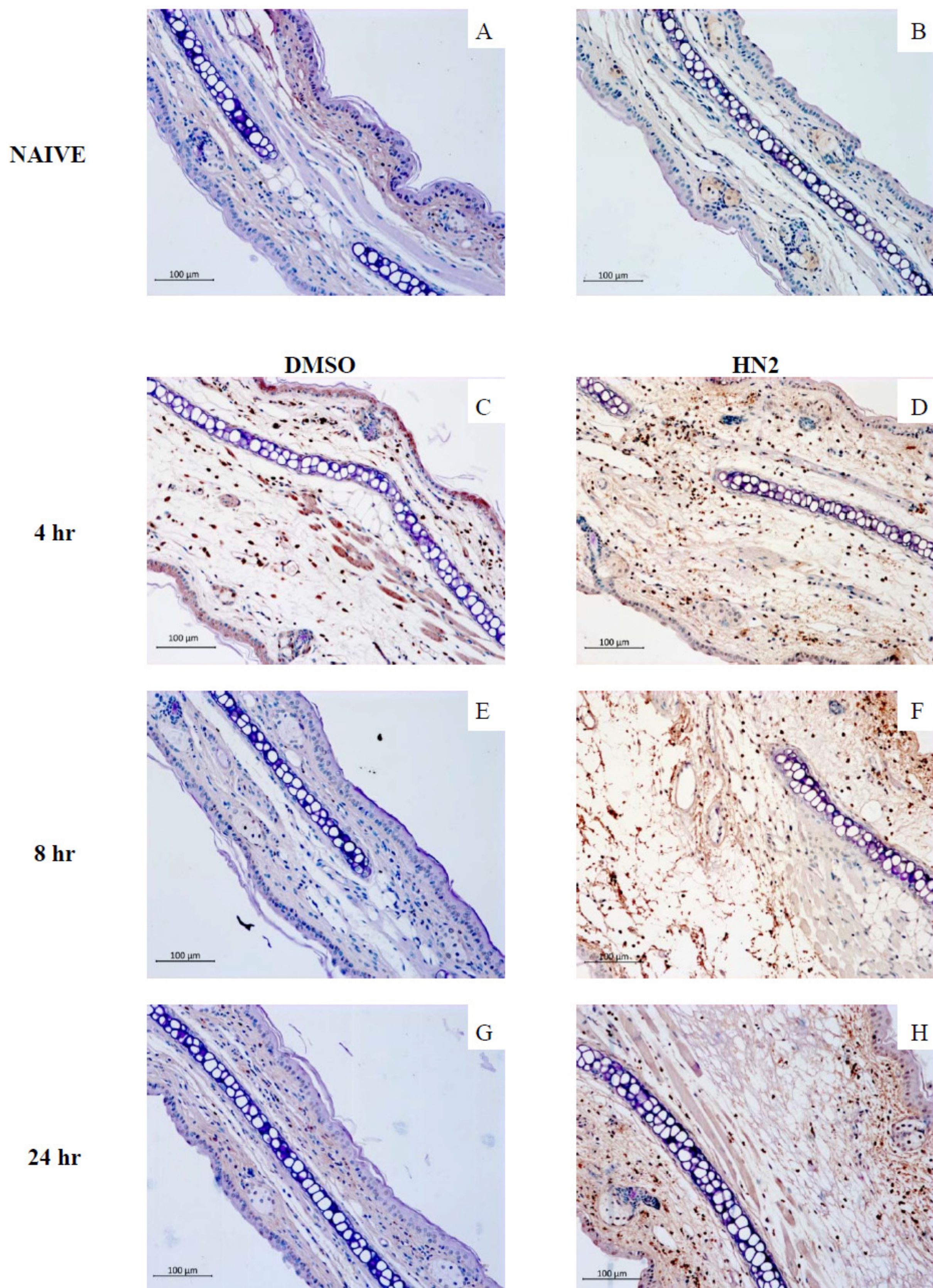


**Figure 6**. Representative light micrographs of IHC staining for MMP-9 in ear tissue sections obtained from male Swiss Webster mice. Panels A and B represent untreated (naïve) ear tissues. Panels C, E and G represent ears treated with the vehicle DMSO only, and Panels D, F and H represent ears treated with HN2 (0.5 µmol/ear) at 4 hr, 8 hr and 24 hr, respectively. Total magnification used is 200×. Scale bars on the micrographs represent a length of 100 µm.

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**Table 1.** Vesication/epidermal:dermal detachments in ear punches obtained from male Swiss Webster mice at 15 min, 1 h, 2 h, 4 h, 8 h and 24 h after topical exposure to HN2 or DMSO.

| Time Point | Treatment | % Vesication | N (# of ears) |
|---|---|---|---|
| 15 min | Naïve (untreated) | 0% | 6 |
| 15 min | DMSO (5 µl/ear) | 0% | 12 |
| 15 min | HN2 (5 µmol/ear) | 16.7% | 6 |
| 1 h | Naïve | 0% | 6 |
| 1 h | DMSO | 0% | 12 |
| 1 h | HN2 | 0% | 6 |
| 2 h | Naïve | 0% | 6 |
| 2 h | DMSO | 0% | 12 |
| 2 h | HN2 | 16.7% | 6 |
| 4 h | Naïve | 0% | 6 |
| 4 h | DMSO | 0% | 12 |
| 4 h | HN2 | 16.7% | 6 |
| 8 h | Naïve | 0% | 6 |
| 8 h | DMSO | 0% | 12 |
| 8 h | HN2 | 66.7% | 6 |
| 24 h | Naïve | 0% | 6 |
| 24 h | DMSO | 0% | 12 |
| 24 h | HN2 | 83.3% | 6 |

15 min, 1 hr, 2 hr, 4 hr and 8 hr following HN2 exposure. Mice ears were also collected at the 24 hr time point after HN2 exposure to serve as a positive control.

To our knowledge, few studies have investigated the dermatotoxicity of HN2 at time points earlier than 6 hr using the MEVM. One recent study (Composto *et al.*, 2018) examined epidermal responses to HN2 applied topically to the dorsal skin of mice. In that study, the time points evaluated included 15 min, 30 min, 45 min, 1 hr, 2 hr, 3 hr, 6 hr, 12 hr and 24 hr. The epidermis of the mice was removed at the respective time points and analyzed for dermatotoxicity by H&E staining, immunohistochemistry of 8-oxo-dG and Western Blot analysis for pH2A.X, poly ADP-ribose (pADPr), heme oxygenase-1 (HO-1) and proteins modified by 4-hydroxyl nonenal (4HNE). The results of that study demonstrated that HN2 exposure led to skin inflammation and edema as early as 12 hr, a significant increase in pH2A.X, pADPr, HO-1 and 4HNE as early as 15 min and an increase in the epidermal expression of 8-oxo-dG by 6 hr. However, no signs of vesication were assessed in that study and the gross injury, as shown by light microscopy of H&E tissue sections, was relatively minor. Thus it appears that our study is the first report of vesication by HN2 at time points as early as 15 min following exposure.

The vehicle used here to dissolve HN2 was DMSO. The tissue penetration of DMSO is attributed to an increase in diffusion through the stratum corneum by disruption of the membrane barrier function. This disruption by DMSO has been hypothesized to result from aprotic interactions occurring with intercellular lipids, the reversible distortion of lipid head groups which allows for a more permeable packing arrangement, and the formation of solvent microenvironments within the tissue that enable the solute to be extracted from the vehicle (Capriotti & Capriotti, 2012). Compared to other vehicles, DMSO has been shown to exhibit a greater solubilizing effect on less soluble agents, and its ability to enhance the penetration of a given drug subsequently allows for a greater concentration of the drug to be delivered to the membrane barrier.

In the present study, DMSO applied topically to the mouse ear as a vehicle control resulted in transient tissue edema, as observed by wet weight and morphometric thickness analyses and light microscopy at 15 min, 1 hr, 2 hr and 4 hr. The swelling of ears exposed to DMSO observed here may be attributed to its initial penetration through the mouse ear skin and absorption into the dermis; however, after 4 hr the ear swelling by DMSO subsides and becomes similar to that which is exhibited by untreated naïve ear tissues. Thus, DMSO vehicle, while not inert, did not interfere with the assessment of inflammation or vesication. Until now, we have not known of this DMSO effect, as our previous studies in the MEVM have been carried out ≥12 hr after HN2 exposure. Although the swelling by DMSO subsides after 4 hr, it may be useful to investigate alternate vehicles that could be used to dissolve HN2 for treatment on the mouse ear tissue. For example, Composto and colleagues (2018) dissolved 20 µmol of HN2 in a solution that was 80% acetone and 20% water; forty times higher than the concentration of 0.5 mol HN2 used in the present study. Despite using a much higher concentration of HN2, no evidence of vesication was demonstrated in their study, which may be attributed to a poor ability of the acetone:water vehicle to distribute HN2 into the skin. If this is correct, then vesication by HN2 may require penetration into the dermis, which is achieved with DMSO, but not with acetone or water. Further study will be required to confirm our hypothesis. In addition, whereas Composto *et al.* (2018) exposed the dorsal skin of mice to HN2 in an acetone:water vehicle, it would be interesting to investigate whether the same HN2 formulation exhibits vesication in the MEVM. The use of an acetone mixture poses the risk of evaporation and precipitation on the epidermal surface, which may impact the accurate penetration of the drug into the skin. The use of DMSO in the present study therefore ensures that the full dose of HN2 is delivered into the mouse ear and allows for the presence of vesication to be evaluated.

The results of our study determined that HN2 causes a significant increase in edema in terms of wet weight and morphometric thickness as early as 15 min following HN2 exposure. It should be noted that while both wet weight and morphometric thickness measurements of HN2-treated ear tissues were significant at all time points, the two assays did not match exactly, as demonstrated by the greater increase in ear wet weight observed at 8 hr than at 24 hr, as opposed to the observation of a greater increase

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in morphometric thickness at 24 hr than at 8 hr. The differences observed between the two assays may point to differences in assay sensitivity which should be taken into consideration; however, the ease of determining tissue wet weight as a preliminary index of tissue edema is a great advantage, especially when compared to the more cumbersome tissue preparation required for H&E staining and morphometric thickness analysis. Thus, both assays are complementary and useful in determining the extent by which HN2 induces ear edema. It is noteworthy that both edema and vesication were observed as early as 15 min following HN2 exposure; with a sharp increase in vesication detected between 4 to 8 hr following HN2 exposure. It can therefore be concluded that a 0.5 µmol/ear dose of HN2 in the MEVM results in a time-dependent tissue injury that is accompanied by significant vesication in as little as 8 hr after HN2 exposure.

Matrix metalloproteinases (MMPs) are capable of degrading extracellular matrix proteins and modifying various non-matrix substrates such as cytokines and chemokines. SM exposure leads to upregulation of MMP-9 in a 3D-skin model (Ries *et al.,* 2009) and also *in vivo* (Shakarjian *et al.,* 2006). In the present investigation, the tissue expression of MMP-9 was increased by DMSO vehicle alone up through 4 hr; however, the tissue expression induced by DMSO was transient, confined within immune cells, and returned to baseline levels by 8 hr after vehicle exposure. On the other hand, ear tissues exposed to HN2 exhibited time-dependent accumulation of MMP-9 positive immune cells within the dermis as well as release of MMP-9 into the extracellular matrix. This also correlated with the high incidence of vesication observed at time points ≥8 hr after HN2 exposure.

The results of this study provide a more holistic understanding of the process of vesication and reveal useful information for future investigation aimed at identifying potential antidotes to mustard toxicity. For example, previous work in our lab has applied various potential antidotes to HN2-exposed ears at 15 min, 4 hr and 8 hr following initial exposure, with tissues harvested at 12 hr and 24 hr following exposure (Tumu *et al.,* 2018). By utilizing the information provided by the present study, it may be useful to apply countermeasure treatments at alternative time points such as 1 hr, 2 hr and 4 hr after HN2 exposure, as significant tissue swelling and inflammation are already observed at these earlier time points. Additionally, future studies may look to euthanizing mice at 8 hr following treatment(s), as the extent of vesication induced by HN2 begins to plateau by this point, and only increases slightly up to 24 hr. Moreover, the present study sets the stage to investigate DNA damage and other factors involved in the process of vesication and early stages in HN2 injury.

Altogether, the data obtained in the HN2 study demonstrate that:

DMSO vehicle is not inert and affects the mouse ear tissue at early time points (15 min, 1, 2 and 4 hr) after topical application, but not at later time points (8 and 24 hr).

The process of vesication begins as soon as 15 min after topical exposure to HN2 and is significantly upregulated between 4 and 8 hr.

High incidence of vesication (>50%) is time-dependent and associated with accumulation of MMP-9 protein expression in mouse ear tissue. Tissue abundance of MMP-9 increases significantly 2 hr after HN2 exposure, showing expression both within immune cells at 2, 4, 8 and 24 hr, as well extracellular expression at 8 and 24 hr.

These data have established a useful platform for mechanistic studies aimed at better understanding the process of vesication by mustards and the role of MMP-9 therein.

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## Declaration

All authors declare no financial activities or financial holdings that could be perceived as constituting a conflict of interest.

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