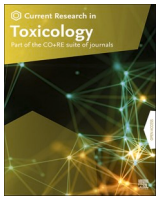
Current Research in Toxicology 7 (2024) 100184

Contents lists available at ScienceDirect

Current Research in Toxicology

journal homepage: www.journals.elsevier.com/current-research-in-toxicology



Investigation of inflammatory mechanisms induced by croton oil in mouse ear

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ARTICLE INFO

Keywords:
Croton oil
Skin
Inflammation
Myeloperoxidase
MMP-9
Dermatotoxicity
MMP-8
PIGN
PIGT
PIGU

ABSTRACT

Croton oil is liquid at room temperature, with a pale-yellow color and spicy odor. It is commonly used in combination with phenol as a chemical peeling agent in dermatology, which reveals its caustic exfoliating effects. Topical use of croton oil at a high dose produces skin irritation, inflammation, swelling, pain, and even tumors. Therefore, croton oil has been widely used for inflammation, pain, and tumor related research, with different animal models having been established. However, mechanistic studies through which croton oil induces skin swelling, injury and activates tissue repair/regeneration are limited. The present study used croton oil to induce mouse ear edema and examined tissue responses 4 h after exposure. To this end, croton oil was applied to the ventral side of mouse ears, followed by tissue collection. Samples were analyzed by hematoxylin and eosin (H&E) staining, toluidine blue staining, and immunohistochemistry staining for myeloperoxidase (MPO) and matrix metalloproteinase-9 (MMP-9). Western blotting and ELISA were also carried out for MMP-9 together with unbiased proteomic analysis using mass-spectrometry. Results from our study demonstrated that as soon as 4 h of exposure to 2.5 % croton oil, the expression levels of MPO and MMP-9 in the dermis significantly increased compared to acetone-treated (vehicle) control ears, as did other inflammatory reactions such as swelling and neutrophil aggregation and infiltration. Subsequently, proteomic analysis confirmed that croton oil treatment resulted in significant upregulation of proteins such as myeloperoxidase (MPO), matrix metalloproteinase-9 (MMP-9), and matrix metalloproteinase-8 (MMP-8) in the ear skin. Interestingly, mouse ears treated with acetone vehicle showed differential expression of 2,478 proteins relative to naïve tissues; among those differentially expressed in acetone-treated samples were members of the phosphatidylinositol-glycan biosynthesis class N, T and U proteins (PIGN, PIGT, and PIGU). Overall, this work confirms the presence of neutrophil-derived MPO and MMP-9 and extends the body of knowledge to show that MMP-8 is also present during croton oil-mediated skin inflammation in the mouse ear; moreover, we find that acetone vehicle is not inert and has effects on the skin that should be considered moving forward.

1. Introduction

Croton oil, referred to as, "bā dòu" in traditional Chinese medicine, is derived from the seeds of the plant *Croton tiglium (L.)* (Niu et al., 2020) and has been used for centuries as a treatment for constipation, due to its strong purgative action on the GI tract, as well as for rheumatism, headache, peptic ulcers and visceral pain (Tsai et al., 2004; Wang et al., 2008). The oil is a complex mixture, containing proinflammatory terpenes (especially diterpenes known as phorbol esters) (Kim et al., 2015; Pagani et al., 2017; Xu et al., 2018) as well as several other components including oleic acid and linoleic acid (Lan et al., 2012). The most abundant phorbol ester constituent of croton oil is 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which is well known for its tumor promoting and skin irritating effects (Ajith and Janardhanan, 2011; Castagna et al.,

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https://doi.org/10.1016/j.crtox.2024.100184
Received 15 January 2024; Received in revised form 12 May 2024; Accepted 21 June 2024
Available online 23 June 2024

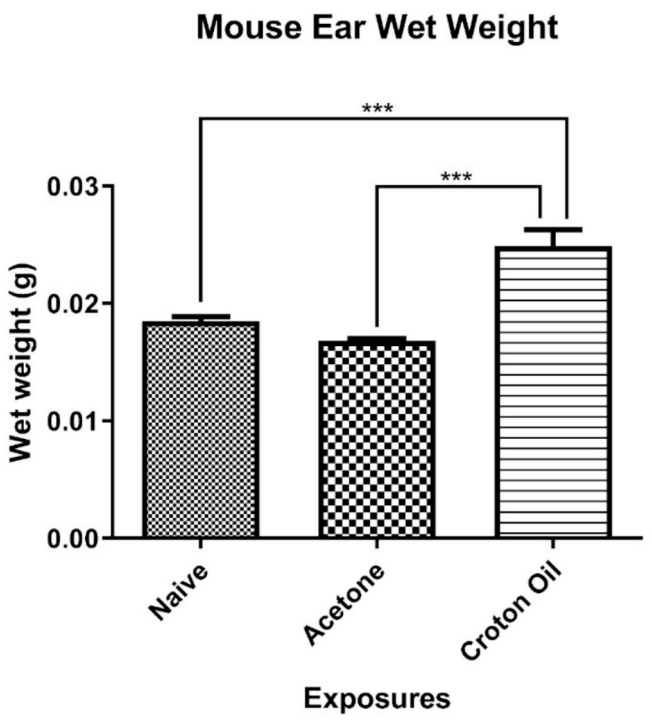

Fig. 1. Wet weight of ear punch biopsies. Data analyzed by One-way ANOVA analysis followed by Newman-Keuls post-hoc analysis. N = 4 independent biological mouse ears/group.

1982; Schmidt and Evans, 1980; Van Duuren and Orris, 1965; Young et al., 1983). When applied to skin in a phenol carrier, croton oil serves as a potent chemical peeling agent that not only reduces pigmentation of skin but also stimulates collagen synthesis within the dermis (Larson, 2005; Wambier et al., 2019). Croton oil is the active ingredient in chemical peeling formulations (Bensimon, 2008) and was selected for investigation by our laboratory over TPA because human exposure to the complex mixture croton oil is more common than to TPA alone.

In modern times, the use of croton oil in humans is predominately relegated to skin peeling and skin rejuvenation in humans. In addition, croton oil is widely used in experimental mouse models of inflammation. In particular, the croton oil ear test induces cutaneous inflammation predominately for the purpose of evaluating new drugs with anti-inflammatory activity (Colorado et al., 1991; De Young et al, 1989; Gábor, 2003; Livingstone et al., 2023; Moon et al., 2001; Prado et al., 2023; Sangchart et al., 2021). While it is well known that cutaneous tissue responses to croton oil evolve over several hours and involve redness, swelling, pain, and infiltration of immune cells; the toxicodynamic tissue responses to croton oil remain to be fully described.

In the present study, we investigated toxicodynamic responses of mouse ear skin 4 h after a single topical exposure to croton oil. We observed that mouse ear skin treated with croton oil exhibited edema, immune cell infiltration (predominately neutrophils), a transition from spindle shaped dermal mast cells to rounded ones, and an overall decrease in mast cell numbers within the skin. We next investigated the effect of croton oil on tissue expression of matrix metalloproteinase-9 (MMP-9) and myeloperoxidase (MPO) in mouse ear skin. Whereas MMP-9 is a proteolytic enzyme capable of degrading extracellular matrix proteins such as collagen (Bigg et al., 2007; Cirillo and Prime, 2021), MPO is another important neutrophil marker involved in several inflammatory and pathological conditions. It has important functions in neutrophil activities such as antisepsis, and it is upregulated under inflammatory reactions and oxidative stress situations (Ndrepepa, 2019) as well as in mouse ear treated with croton oil at time points later than 4 h (Cardia et al., 2018; Valverde et al., 2021). Using immunohistochemistry (IHC), we found that both MMP-9 and MPO were increased in mouse ear skin 4 h after exposure to croton oil. Proteomic analysis of ear skin tissue extracts revealed that matrix metalloproteinase-8 (MMP-8), MMP-9 and MPO were among the 1,860 differentially expressed proteins induced by croton oil (relative to ear tissues exposed only to acetone vehicle). It is worthy to note that acetone exposure (the vehicle) was not inert and resulted in > 2,000 differentially expressed proteins (relative to naïve ear tissues). Taken together, the present study represents an important contribution to the current understanding of cutaneous responses to croton oil.

2. Materials and methods

2.1. Test Compounds

Acetone was obtained from VWR (Cat# 67-64-1) and served as the vehicle in this study. Croton oil was purchased from VWR (Cat # TCC0421-025ML).

2.2. Chemicals, reagents, kits and other materials

Isoflurane (Cat # 029405) was purchased from Henry Schein (Dublin, OH). Permount (Cat # SP15-500) and slides (Cat # 12-544-2) were purchased from Fisher Scientific (Fairlawn, NJ). Neutral buffered formalin (1:10 dilution, already diluted) (Cat # 23-245685) was purchased from Fisher Scientific (Nazareth, PA). Cover glasses (Cat # 48382-136), eosin (Cat # 95057-848), hematoxylin (Cat # 95057-844), xylene (Cat # 89370-088), histology grade 100 % ethanol (Cat # 89370-084), Tris Buffered Saline (TBS) (10X) (Cat # 101642-634) and Paraplast X-tra (Cat # 15159-486 −1 kg) were purchased from VWR International (West Chester, PA). Tween-20 (Cat # 97062-332) and methyl green, zinc chloride salt (Cat # 97061-398) was purchased from VWR International (Solon, OH). 30 % hydrogen peroxide (H_2O_2) was purchased from VWR International (Mississauga, ON, Cat # BDH7690-1). 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 # EM-6505). The 100 % n-butanol was purchased from EMD Millipore (Billerica, MA; Cat # BX1777-6). The myeloperoxidase (MPO) (Cat # ab208670), matrix metalloproteinase-8 (MMP-8) (Cat # ab53017), and matrix metalloproteinase-9 (MMP-9) (Cat # ab38898) primary antibodies were purchased from Abcam (Boston, MA).

2.3. Animals

Male Swiss Webster mice (25–30 g) 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). All animals were housed in groups of 2–4 per cage in temperature and humidity regulated rooms with 12 h − day and 12 h − night cycles. Animals were allowed to adjust to the new environment for at least 2–3 days before use. All mice had access to food and water *ad libitum*. 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).

2.4. Croton oil ear inflammation model

When mouse ear skin is exposed to croton oil, robust cutaneous responses including edema, redness and immune cell infiltration are observed within a 24 h period (Gábor, 2003).

2.4.1. Test solutions and reagents

(a) Croton oil solution: A 26.6 μL volume of croton oil (density: 0.94

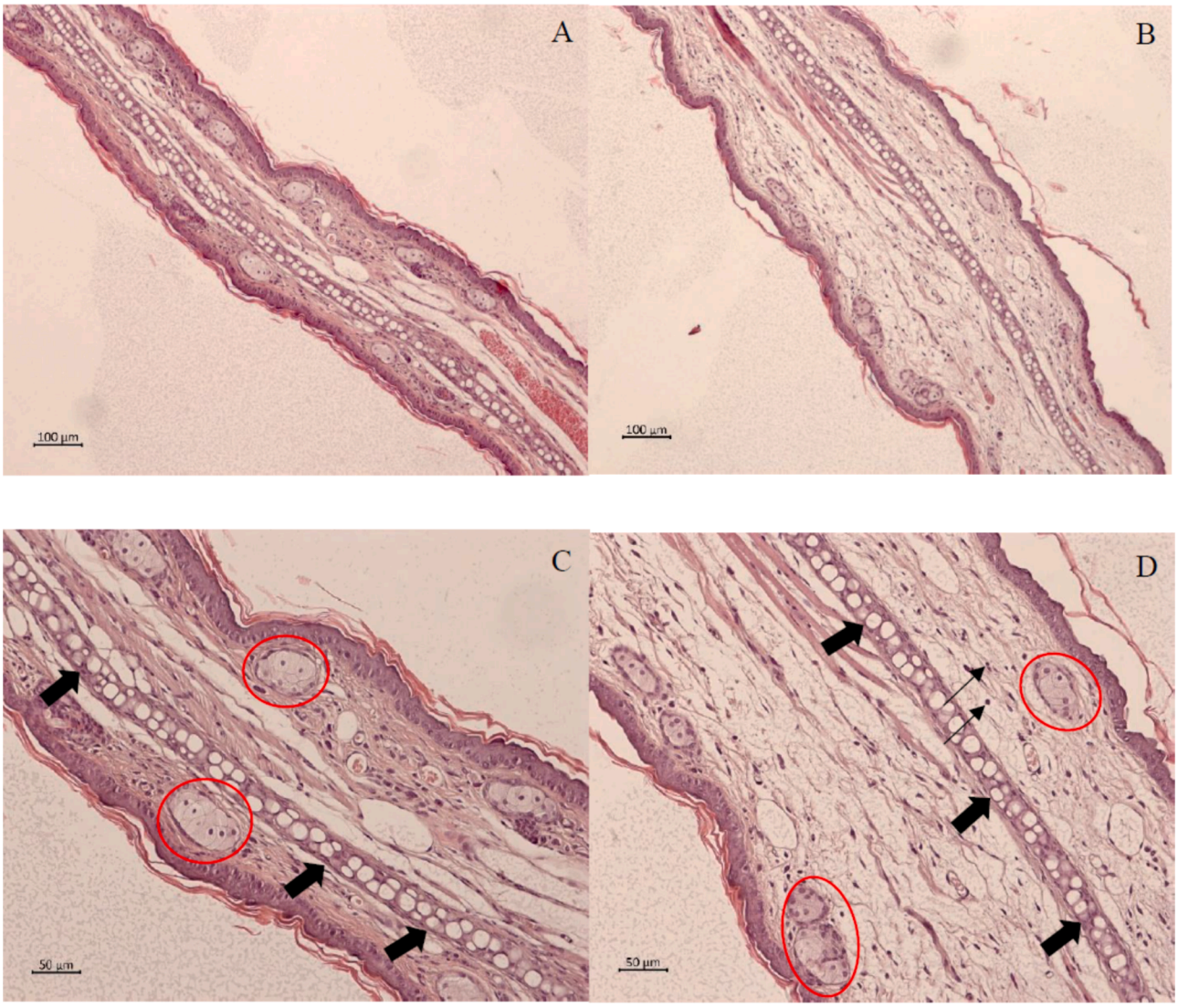

Fig. 2. H & E staining. Panel A: acetone treated mouse ear, 100x; panel B: croton oil-treated mouse ear, 100x; panel C: acetone treated mouse ear, 200x; panel D: croton oil-treated mouse ear, 200x. The red circles indicate sebaceous glands, the bold arrows indicate cartilage, and the thin arrows indicate neutrophils. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

g/mL) was dissolved in 973.4 µL of acetone to obtain a 2.5% solution of croton oil and well mixed. During topical exposure, 10 µL of this solution, which equals to 0.266 µL of pure croton oil, was administered to the inner surface of a mouse ear. We chose and adjusted to this concentration because a similar dose was used by another laboratory to significantly induce ear swelling in Swiss Webster mice (Colorado et al., 1991).

(b) Other reagents. Vehicle: 10 µL of acetone were applied to the ears that served as vehicle (control) tissues. Buffered formalin solution (1:10 dilution): Neutral buffered formalin 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 solution purchased from VWR was used to stain the nuclei of the tissue sections. Eosin staining solution: Ready-made eosin solution purchased from VWR was used to stain the cytoplasm of the tissue sections. Toluidine Blue (Cat # BP107-10) was obtained from Fisher Scientific (Fairlawn, NJ).

2.4.2. Experimental design

Wild type, male Swiss Webster mice weighing 25–30 g were separated into groups. The right ears were treated with a 10 µL volume of 2.5 % (wt/vol) croton oil solution in acetone (0.266 µL pure croton oil), while the left ears served as test controls and received either a similar volume of acetone (vehicle ears) or were left untreated (naïve ears). Four hours after the exposure, animals were euthanized using carbon dioxide (CO_2) and ear tissue samples were collected using 8 mm biopsy punches. The ear samples were weighed and then transferred to 20 ml vials with 10 ml neutral buffered formalin for 24 h before dehydration, embedding in paraffin, tissue sectioning and H&E staining as described previously (Tumu et al., 2020) or Toluidine Blue staining as described by Lee and colleagues (2021).

2.5. Western blotting for MMP-9

2.5.1. Tissue harvesting for Western blotting

Ear tissues were harvested from the mouse by use of an ear punch and then preserved in RIPA buffer containing a cocktail of protease inhibitors. The tissues were then homogenized by use of a sonicator to form lysates of the different samples, which were then stored at −80 °C to prevent degradation.

The tissue lysates were thawed and assessed for protein content using

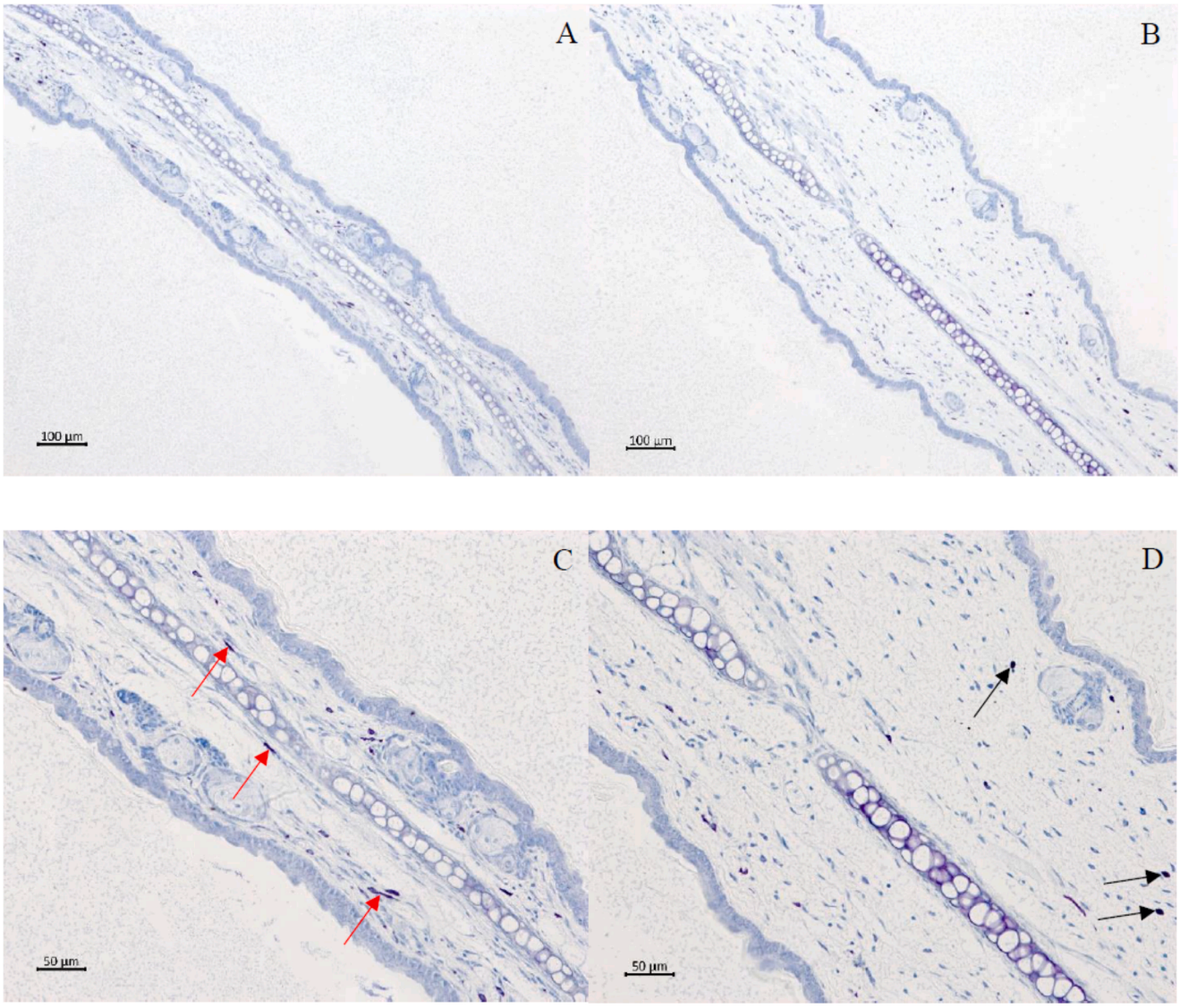

Fig. 3. Toluidine blue staining. Panel A: acetone treated mouse ear, 100x; Panel B: croton oil-treated mouse ear, 100x; Panel C: acetone treated mouse ear, 200x; Panel D: croton oil-treated mouse ear, 200x. The red arrows indicate spindle shaped mast cells, and the black arrows indicate round mast cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Scoring of the toluidine blue stained sections.

Exposure	# of cells/ear section (mean ± SEM); N = 3 ears
Acetone	86.88 ± 3.13
Croton Oil*	63.75 ± 1.00

*The number of mast cells is significantly different between acetone and croton oil-treated mouse ears, according to a student *t*-test ($p<0.05$).

the BCA protein assay. Next, 2 mcg/sample was mixed with a loading buffer and heated at 95 °C for 5 min to denature the proteins. All samples were then loaded onto SDS page gels and ran for 1 h at 100 V. Each gel was then placed into the apparatus for Western blotting along with a membrane paper soaked in transfer buffer. The apparatus was then run for 90 min at a constant ampere of 200 mA. Each PVDF membrane paper was removed and blocked overnight with 5 % nonfat milk. After rocking the blot for 30 min at room temperature, the primary antibody for MMP-9 was added to the container and rocked for 3 h. Then the primary antibody was removed, and blots were washed with TBS-Tween 20 for four 12-minute washes. Then the secondary anti-rabbit IgG HRP-linked antibody was added to a mixture of 4.5 mL of TBS-Tween 20 and 5 % milk, vortexed and added to the blots to shake for 1 h. The secondary antibody was removed and then the membranes were washed again with four 12-minute washes with TBS-Tween 20. Blots were then imaged by chemiluminescence using a PICO reader mixture of 9 mL.

2.6. ELISA for MMP-9

An ELISA assay was performed for MMP-9 using a mouse MMP-9 kit (R&D Systems, Cat # MMPT90). First, 100 µL of sample or standards (diluted in reagent diluent) were added to each well and incubated overnight at 4 ℃. The next day, wells were aspirated and then washed using a wash buffer for six 5-minute washes. Then 100 µL of the detection antibody was added to each well and incubated for 2 h at room temperature. The wash step was then repeated. Next, 100 µL of the working dilution of Streptavidin-HRP was added to each well and incubated for 20 min at room temperature while avoiding direct light. The wash step was repeated again, after which 100 µL of substrate solution was added to each well to incubate for 20 min at room

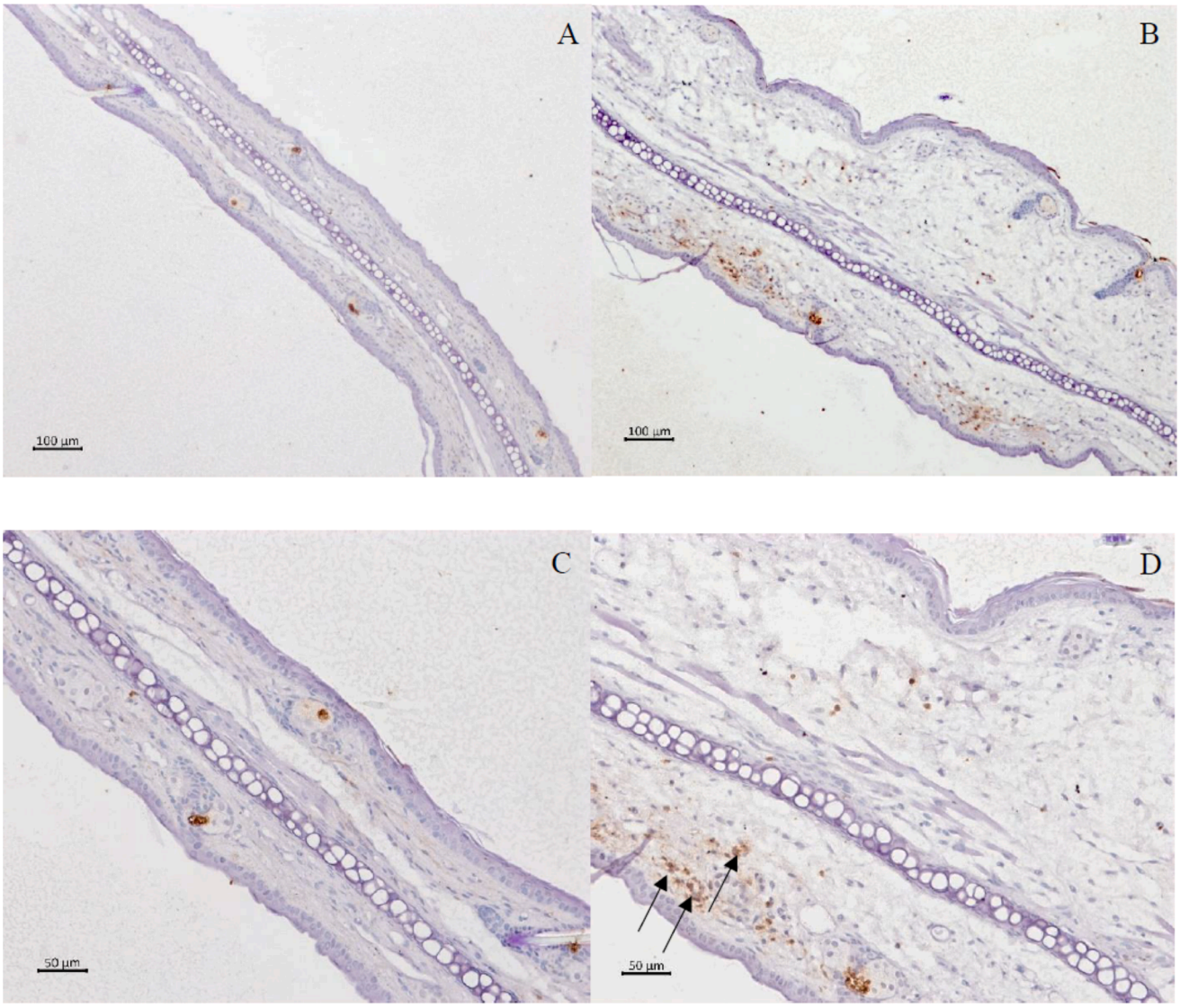

Fig. 4. IHC staining for MPO. Panels A and C: MPO staining on acetone exposed mouse ear, 100x and 200x; Panels B and D: MPO staining on croton oil exposed mouse ear, 100x and 200x. Arrows indicate MPO positively stained cells.

temperature (avoiding direct light). 50 µL of stop solution was then added to each well and stirred with a pipet tip. The plate was then read at 450 nm and corrected wavelength at 540 nm.

2.7. Immunohistochemistry (IHC) for MMP-8, MMP-9 and MPO

Unless otherwise indicated, IHC was carried out as described previously (Tumu et al., 2020). Note that the primary antibody dilution for MMP-8 was 1:50.

2.8. Proteomic analysis of mouse ear tissue homogenates

Equal amounts of ear tissue extracts (10 µg) from naïve, acetone-treated, or croton oil-treated mouse ears were subjected to SDS-PAGE (100 V, 10 min). Each lane represented a different mouse ear extract with 4 different mouse ears (4 lanes of the gel) run for each treatment, and a total of 3 treatment groups (naïve, acetone-treated or croton oil-treated) (12 lanes altogether). The gel was stained with Coomassie Blue overnight. The following day, bands were excised. Tryptic digests were analyzed using an Orbitrap Tribrid mass spectrometer and nano-flow LC system (Thermo Scientific) as described in Barth et al. (2021). The raw liquid chromatography–mass spectrometry data were converted into MASCOT Generic Format using Proteome Discover 2.41 (Thermo Fisher) and searched against either the Uniprot mouse proteome database or National Center for Bio-technology Information (NCBI) mouse database together with a database of common laboratory contaminants (https://www.thegpm.org/crap/) using a local implementation of the global proteome machine (Beavis, 2006). Raw counts were used to calculate the %abundance for each GPM-identified protein. Proteins with p-values < 0.05 and absolute log2 fold changes > 1 were called as differentially expressed. Comparisons were made between naïve control vs. vehicle-treated (acetone) ear tissues, naïve control vs. croton oil-treated ear tissues, and vehicle-treated vs. croton oil-treated ear tissues. Volcano plots were generated using GraphPad Prism (Version 10.1.1). Venn diagrams were generated using bioinformatics.psb.ugent.be/webtools/Venn/. Heatmaps of raw counts were generated using Morpheus at https://software.broadinstitute.org/morpheus/.

2.9. Statistical analysis

Unless otherwise indicated, all the results were reported as mean ± SEM for 4 samples. Statistical significance was tested between groups using either a student *t*-test or a one-way ANOVA followed by post hoc analysis and GraphPad Prism® version 5.0 software. R analysis was

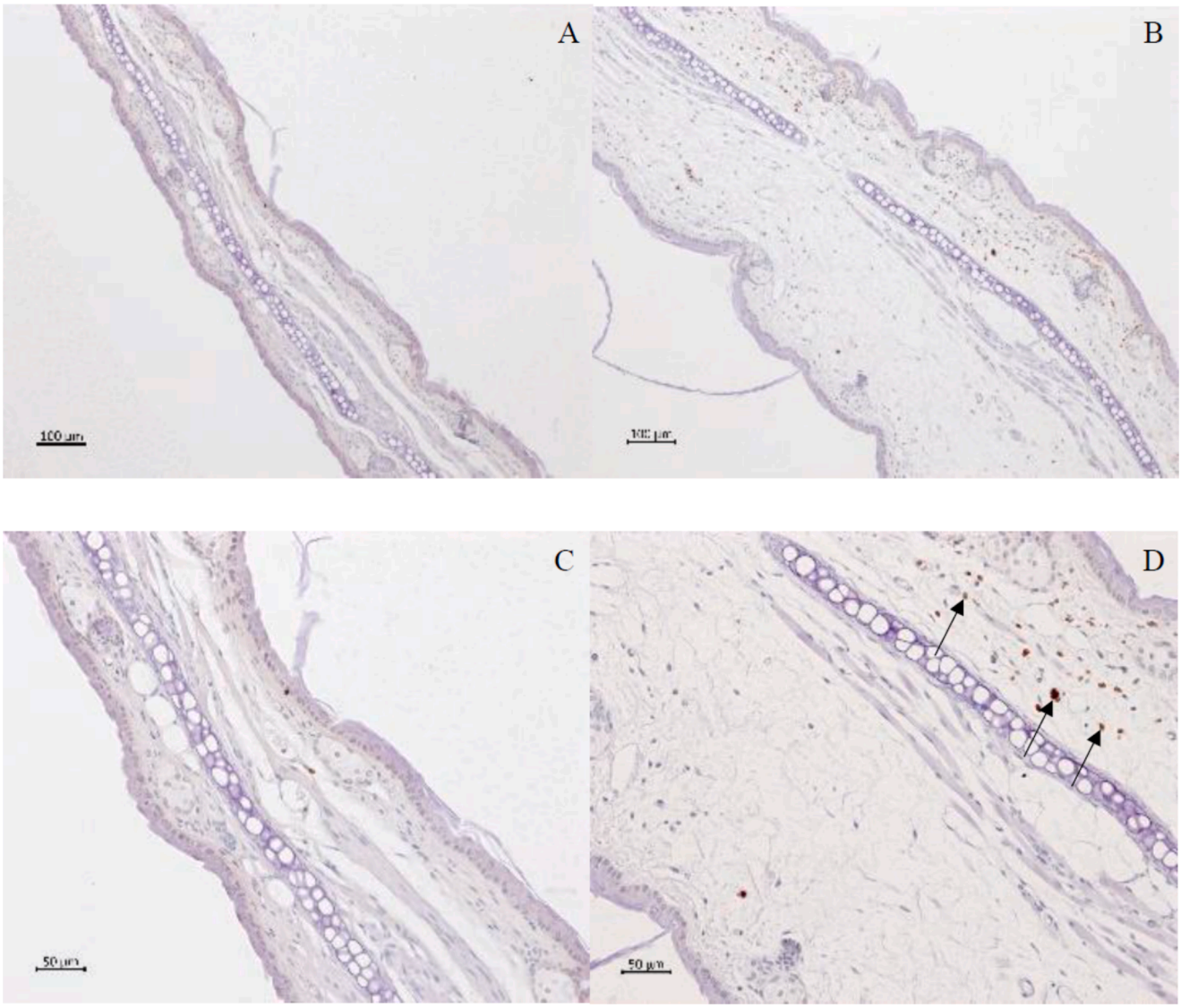

Fig. 5. IHC staining for MMP-9. Panels A and C: MMP-9 staining on acetone exposed mouse ear, 100x and 200x; Panels B and D: MMP-9 staining on croton oil exposed mouse ear, 100x and 200x. Arrows indicate MMP-9 positively stained cells.

carried out to analyze the mass spectrometry data as described by Barth et al. (2021).

3. Results

3.1. Croton oil and ear edema

First, croton oil was used to induce ear edema. To this end, the inner surface of the right ears of male Swiss Webster mice were treated topically with croton oil (2.5 % wt/vol, 10 μL/ear). Left ears served as either naïve or vehicle control which received an equivalent volume of acetone only. Vehicle and croton oil-treated mouse ears were harvested 4 h after exposure. To this end, mice were euthanized with CO_2 and ear punch biopsies (8 mm each) were obtained and weighed. Compared to untreated ears (18.5 mg), ears exposed to acetone were similar in weight (16.8 mg). However, ears treated with croton oil (24.9 mg) were ~ 1.5 fold heavier than control and vehicle ears, respectively (Fig. 1).

3.2. Effects of croton oil on ear tissue histology

Light micrographs revealed that H&E-stained ear sections obtained 4 h after topical exposure to acetone appeared normal, with two visible epithelial surfaces, evenly dispersed sebaceous glands and a prominent cartilage layer within an otherwise unremarkable dermis (Fig. 2, Panels A and C). By 4 h after exposure to croton oil, significant ear tissue swelling, and edema was observed (Fig. 2, Panels B and D) along with an infiltration of immune cells and disaggregated collagen in the dermis.

Toluidine blue staining of mouse ear sections demonstrated that spindle-shaped mast cells were present in the dermis of acetone-treated ears (Fig. 3, Panels A and C) but that the cells changed morphology and became rounded 4 h after exposure to croton oil (Fig. 3, Panels B and D).

In addition, 4 h after croton oil exposure, the number of mast cells in the dermis was found to be decreased relative to vehicle-treated ear tissues (Table 1).

3.3. Effects of croton oil on inflammatory markers

IHC analysis for the presence of the neutrophil markers myeloperoxidase (MPO) and matrix metalloproteinase 9 (MMP-9) was carried out on all the mouse ear sections. In mouse ears collected 4 h after acetone exposure, background MPO staining in sebaceous glands was observed (Fig. 4, Panels A and C). However, in mouse ears collected 4 h after croton oil exposure, an increase in MPO+ immune cells was observed, especially in the dermis of the same side to which the croton oil was

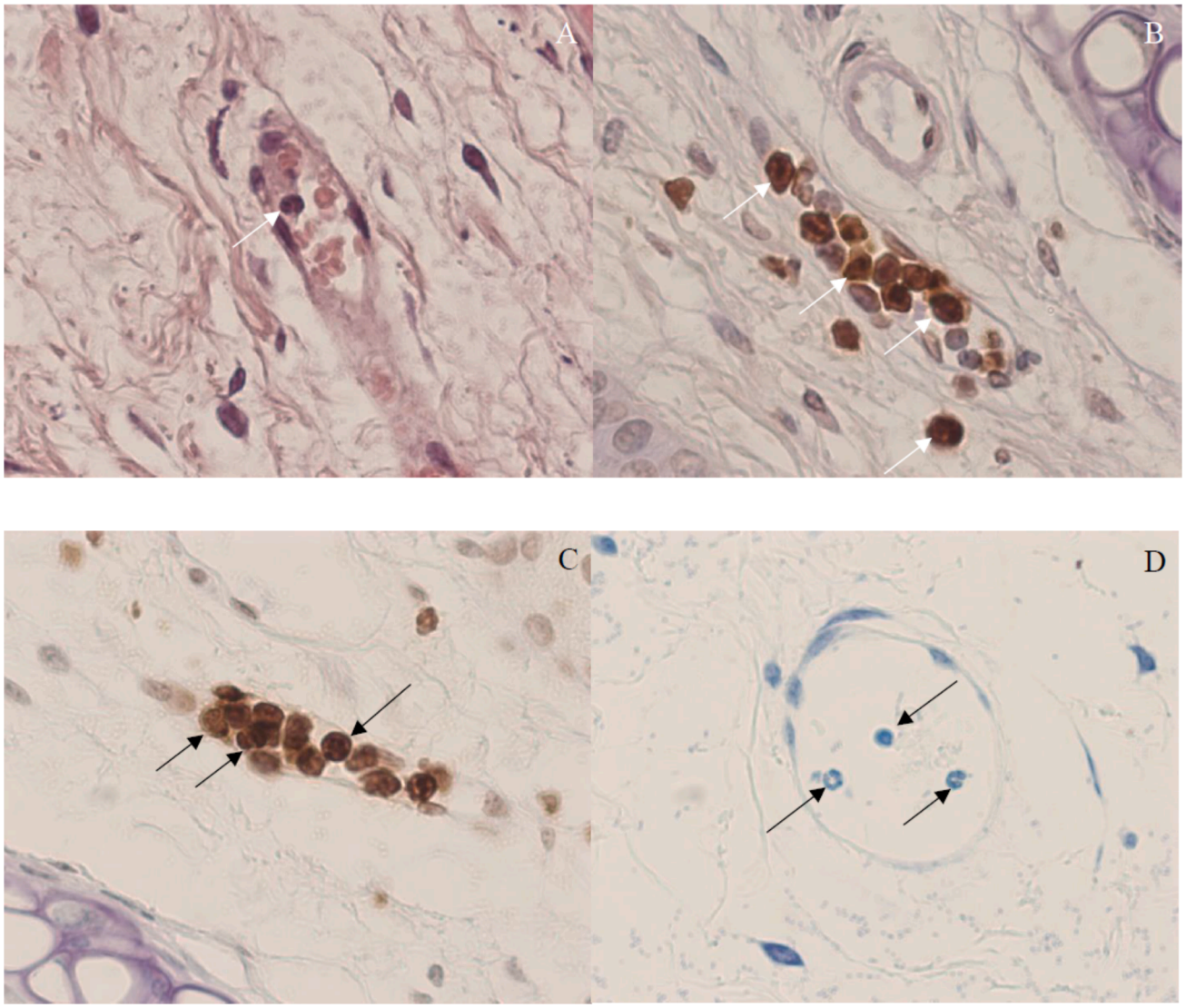

Fig. 6. Neutrophils in different staining, under 1000x magnification. The arrows indicate the neutrophils, which are mostly found in vessels. Panel A: H & E staining; Panel B: MMP-9 IHC staining; Panel C: MPO IHC staining; Panel D: toluidine blue staining. White and black arrows both indicate neutrophils in different stainings. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

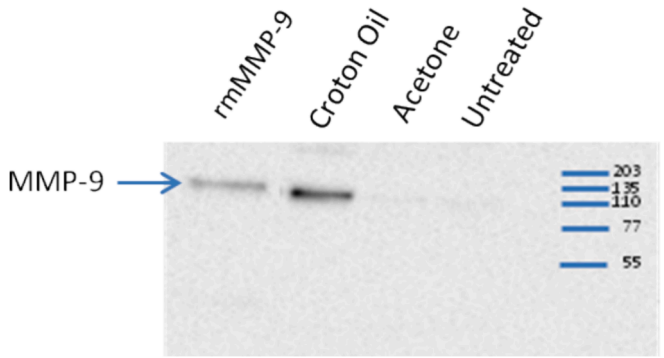

Fig. 7. Western blot for MMP-9. Mouse ear tissue lysates (2 μg/well) were run on SDS-PAGE and transferred to PVDF membrane and probed for MMP-9. Ear skin treated with croton oil showed a strong upregulation of MMP-9 compared to acetone-treated or untreated ear tissues. Note that recombinant mouse MMP-9 (rmMMP-9; 37.5 pg, Lane 1) was loaded on the gel as a positive control.

applied (ventral surface) (Fig. 4, Panels B and D).

Similarly, mouse ears treated with acetone vehicle showed low to minimal brown staining within the sebaceous glands (Fig. 5, Panels A and C) while ears treated with croton oil showed an abundance of MMP-9 + immune cells in the dermis of the treated side (Fig. 5, Panels B and D).

Oil-immersion light microscopy (1000x) of H&E-stained tissues revealed that neutrophils were the predominant infiltrating immune cells in croton oil-treated ears (Fig. 6, Panel A) and cells expressing MPO or MMP-9 are also neutrophils (Fig. 6, Panels B and C, respectively). The tripartite nucleus is also seen clearly in Fig. 6, Panel D.

Mouse ear tissue lysates were loaded onto SDS-PAGE gels and transferred to PVDF membranes for Western Blotting for MMP-9. Protein bands corresponding to MMP-9 were detected in croton oil-treated samples but not in acetone-treated or naïve tissues (Fig. 7).

Moreover, an ELISA for MMP-9 showed similar results, with croton oil-treated ears showing an approximately 5-fold significant increase in ear tissue lysates relative to acetone-treated or naïve tissues (Fig. 8).

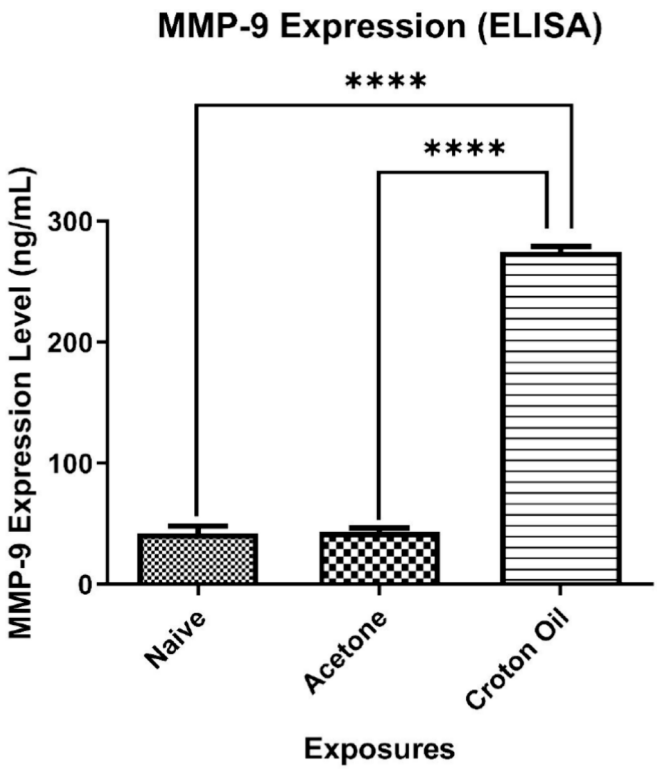

Fig. 8. MMP-9 ELISA. One-way ANOVA analysis followed by Tukey *t*-test. N = 4 independent biological mouse ears/group.

3.4. *Investigating the differential expression of cutaneous proteins in naïve, acetone, or croton oil-treated mouse ear skin*

Proteomics analysis was performed to investigate the differential expression of proteins in naïve ear tissues versus ear tissues biopsied 4 h after exposure to either acetone or croton oil. To identify differentially expressed proteins by croton oil, we employed the following stepwise approach: First, we prepared Venn diagrams (Fig. 9A) for the list of proteins identified as differentially expressed for 'naïve vs vehicle' and 'naïve vs treated' groups. This led us to identify: 858 differentially expressed proteins (DEP) that are unique to acetone (vehicle) treatment, 1620 DEPs that are common to vehicle and croton oil treatment and 1860 DEPs that are unique to croton oil treatment. Next, we prepared a volcano plot comparing naïve vs acetone-treated tissues (Fig. 9B). It is noteworthy that ear tissues treated only with acetone (vehicle) revealed 2,478 (858 + 1620) proteins that were differentially expressed compared to naïve tissues, of which 101 proteins were downregulated and 2,377 were induced (Fig. 9, Panel B, blue dots). Among the proteins induced by acetone were members of the phosphatidylinositol glycan (PIG) family and included PIGN, PIGT, and PIGU (Fig. 9, Panel B, blue dots; Supplemental Table S1). Next, we prepared a volcano plot to compare naïve vs croton oil-treated tissues (Fig. 9, Panel C). When naïve mouse ear tissues were compared to croton oil-treated ears, 3,480 proteins were identified as differentially expressed, with 1,620 of those proteins common to both vehicle and croton oil-treated groups (Fig. 9, Panel C, blue dots) and 1,860 proteins differentially expressed in croton oil-treated ear skin, of which 328 were upregulated and 1532 were downregulated (Fig. 9, Panel C, green dots). The proteins differentially expressed in croton oil-treated skin included matrix metalloproteinase-8 (MMP-8), MMP-9 and MPO (Fig. 9, Panel C, green dots; Supplemental Table S2). This stepwise analysis was cross verified by preparing heatmaps of raw counts of the top 100 differentially expressed proteins from Fig. 9C (Fig. 9, Panel D). When acetone-treated ear skin was compared to croton oil-treated skin, no differential expression of PIGN, PIGT, or PIGU was observed (Supplemental Table S3), indicating that their induction was triggered by topical exposure to acetone alone while MMP-8, MMP-9 and MPO were only found to be increased in the croton oil-treated ear skin. This supports the IHC data presented earlier (Figs. 4 and 5). An additional IHC study was carried out to confirm the tissue expression of MMP-8 in croton oil-treated samples (See Supplemental Fig. S1).

3.5. *Summary of results*

A summary of findings for the 4 h study is described in Table 2. It is worthy to note that the effect of croton oil at 4 h included increased tissue wet weight (edema), immune cell infiltration and expression of the neutrophil markers MPO, MMP-8, and MMP-9. To our knowledge, the discovery of the presence of MMP-8 in croton oil-induced skin inflammation has not been reported previously. Moreover, mast cells in the dermis changed from a spindled shape to a rounded shape and decreased in number 4 h after croton oil exposure.

4. Discussion

Croton oil is a complex lipid mixture that contains multiple molecules that add to the severity of its inflammation (Rohrschneider, et al., 1972). Croton oil contains phorbol ester, which activates protein kinase C and other inflammatory mediators, promoting irritant and cutaneous signs similar to psoriasis (Ferreira et al., 2023). We used the croton oil model because it creates a fast and immediate reaction on the skin, triggering local inflammation, inducing edema and erythema, also increasing vascular permeability, leukocyte infiltration observed in blood vessels within the ear, synthesis of eicosanoids and liberation of nitric oxide (Cardia et al., 2018; Swingle et al., 1981). While there are numerous reports of the effects of TPA, a major component of croton oil, on skin inflammation (Ajith and Janardhanan, 2011; Castagna et al., 1982; Schmidt and Evans, 1980; Van Duuren and Orris, 1965), we chose to investigate croton oil over TPA as human exposure to croton oil is more likely than to TPA primarily because croton oil is still used in combination with phenol as a chemical peel in humans. On the other hand, pure TPA is often used for research involving tumor development and is unlikely to be used in common cases of inflammation.

Previous results from other researchers have shown that in the mouse model, topical exposure to croton oil induces neutrophil aggregation (Kitajima et al., 2018). Meanwhile, previous research has indicated that polymorphonuclear neutrophils (PMNs) release different granular enzymes and/or mediators, including myeloperoxidase (MPO) and metalloproteinase-9 (MMP-9), etc., under inflammatory situations (Modestino et al., 2023). As to the MMP-9, it is significantly upregulated after long term topical exposure to croton oil, up to 16 weeks (Subramanian et al., 2014). Yet, few studies focus on the short term MMP-9 level changing after croton oil exposure. In our IHC staining results, we found significant upregulation of both MPO and MMP-9 in the polymorphonuclear cells, where the cell and nucleus shape suggests neutrophils. Other researchers have also shown that 24 h after croton oil exposure, both PMN total number and the proportion of PMNs to total leukocytes are promoted in the exposed site, and that this effect can persist for a longer time like 2–3 days (Weber et al., 2015). Our data are in line with that observation and demonstrate the aggregation of PMNs in the blood vessels and dermis, with upregulation of MPO and MMP-9 emerging as early as 4 h after topical exposure to croton oil. Several groups have examined the effect of croton oil on mouse ear at 6 or 24 h after exposure (Cardia et al., 2018; Ferreira et al., 2023; Valverde et al., 2021), but we investigated the tissue response at 4 h after exposure. Pinto and colleagues (2015) observed intense vasodilation and the presence of inflammatory cytokines including IL-1, TNF-alpha, and IL-6 in mouse ear tissues obtained 4 h after topical application of croton oil. Silva-Filho and colleagues (2015) and Wilches and colleagues (2015) have each independently observed increased MPO activity in ear tissues isolated 4 h after croton oil exposure, but neither group visualized the

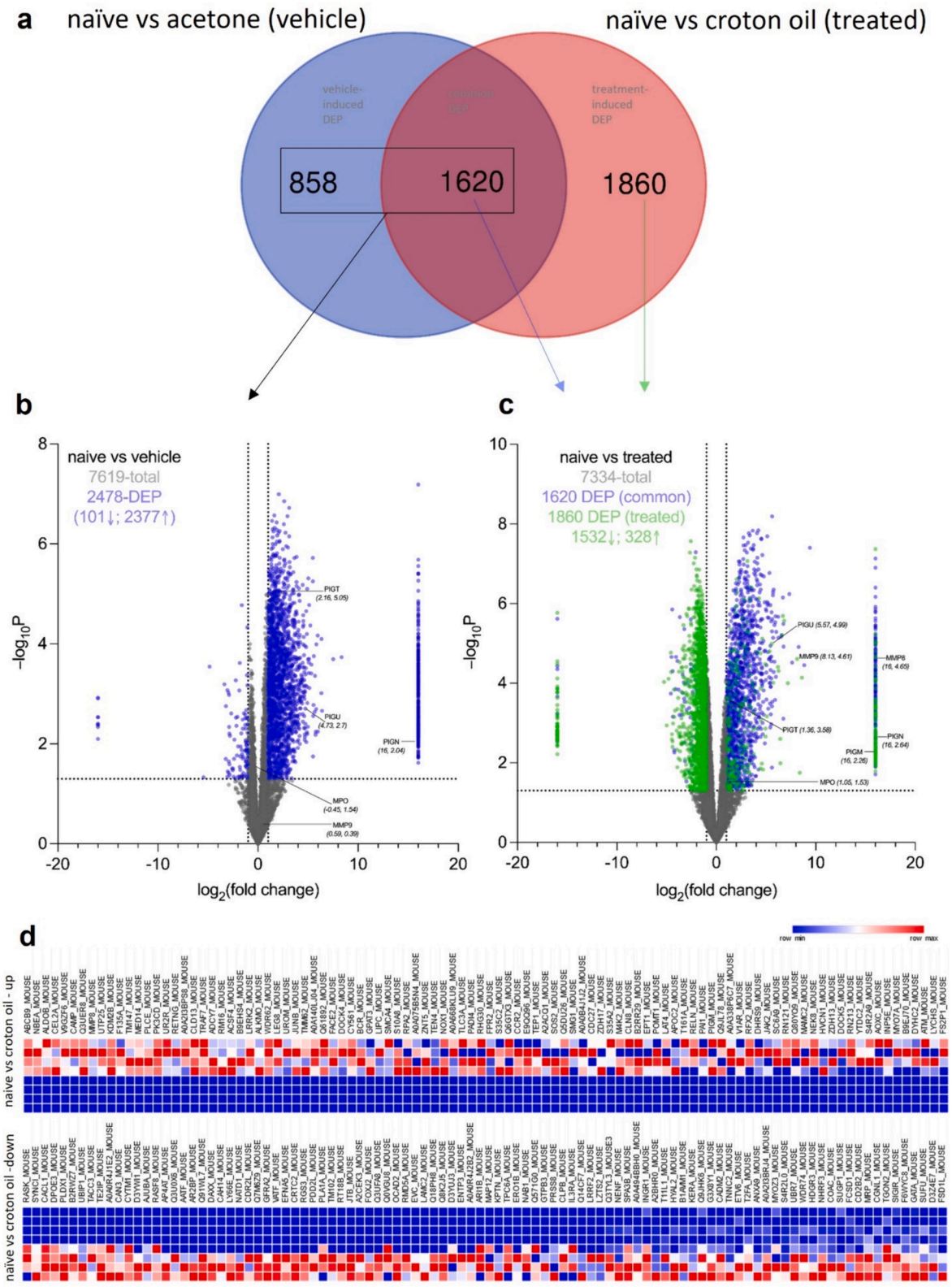

Fig. 9. Effects of acetone and croton oil treatment on the expression of proteins in mouse ear skin. Panel A: Venn diagram comparing proteins differentially expressed between 1) naïve vs acetone vehicle and 2) naïve vs croton oil. Panel B: Volcano plot of relative abundance of proteins in naïve (n = 4) and vehicle-treated mice (n = 4) as measured by mass spectrometry. Dashed line indicates cut-off to identify differentially expressed proteins (p-values < 0.05 and absolute log2 fold changes > 1); blue and black circles indicate points with $p < 0.05$ and $p > 0.05$ respectively. Proteins of interest were labeled with x, y co-ordinates in parenthesis. Panel C: Volcano plot of relative abundance of proteins in naïve (n = 4) and croton oil-treated mice (n = 4) as measured by mass spectrometry. Dashed line indicates cut-off to identify differentially expressed proteins (p-values < 0.05 and absolute log2 fold changes > 1). Blue dots indicate 1,620 differentially expressed proteins that are common between acetone only exposure and croton oil treatment. Green dots are differentially expressed proteins unique to croton oil exposure. Black dots are non-differential expressed proteins. Proteins of interest were labeled with x, y co-ordinates in parenthesis. Panel D: Heatmaps showing raw counts of top 100 differentially expressed proteins (upregulated (top) and downregulated (bottom)) identified as unique to the croton oil-treated group.

Table 2
Summary of the ear tissue effects induced by croton oil.

Parameter	Croton oil effect relative to vehicle
Ear Tissue Wet Weight	↑↑
Infiltrating PMNs	↑↑↑
Mast Cells (Spindle to Round Morphology Transition)	↑
MPO IHC	↑↑
MMP-8 IHC Micrographs	↑
MMP-9 IHC Micrographs	↑↑
MMP-9 Western Blotting	↑↑
MMP-9 ELISA	↑↑↑↑
Proteomic Analysis	↑MPO, ↑MMP-8, ↑MMP-9

tissue localization of MPO using IHC as was done in the present work. Therefore, the present findings for MPO complement those reported in these previous studies, but also extend those findings by including the tissue localization of MMP-9 in response to croton oil at 4 h after exposure. Iba et al. (2007) utilized TPA, the major component of croton oil, and reported that MMP-9 activity increases in a time-dependent manner after topical exposure, but their work was done using tissue homogenates and MMP-9 activity assays while our study also examined the tissue localization of MMP-9 using IHC, again complementing but extending their initial studies.

Regarding the role of mast cells in the response to croton oil, we observed a change in the morphology of toluidine blue stained cells, from spindle shaped to rounded 4 h after exposure; however, the significance of this change will require additional investigation. It is noteworthy to add that an older study found that after subcutaneous injection with croton oil, the number of mast cells in the subcutaneous tissue increased after 24 h and peaked on the 11th day (Feher et al., 1971). In contrast, we observed that there was a decrease in the amount of mast cells in the dermis 4 h after topical exposure to croton oil. The significance of this observed decrease as well as the contributory role of mast cells in the cutaneous response to croton oil remains elusive and warrants further investigation. It is possible that release of mast cell mediators such as mast cell carboxypeptidase (P15089) occurs prior to the 4 h time point and contributes to both the decrease in cell number and the change in morphology from spindle shaped to rounded, but this hypothesis should be further developed and studied. We did observe that mast cell carboxypeptidase was increased in croton oil-treated ear skin relative to acetone treated ear skin (P15089, Log2ratio = 2.59, p = 0.044) but was decreased in acetone-treated ear skin relative to naïve skin (Log2ratio = -2.92, p = 0.022). This switch in carboxypeptidase protein expression from, “decreased by acetone” to “increased by croton oil” is certainly worthy of future study.

One of the most significant disadvantages of the present work is that it examines response to croton oil at a single time point (4 h after exposure). Yet, it is important to note that the status of both tissue injury and the commencement of tissue repair is likely to change as a function of time. To acquire a more detailed picture of how the expression level of the cell markers changes after croton oil exposure, different exposure times should be investigated. Our studies also tried to interpret the mechanisms of and connections between tissue inflammation and matrix injury (swelling). The present work demonstrated the link between neutrophils, MPO and MMP-9. Previous studies have also shown the degradative effects on skin matrix components such as collagens caused by MMP-9 (Bigg et al., 2007; Jacintho et al., 2018). However, whether MMP-9 is directly upregulated by the MPO or by neutrophil itself and whether inhibition of MMP-9 is protective to tissue matrix or promoting recovery from croton oil-induced tissue injury still requires further study. In addition, here the staining of MPO and MMP-9 were each completed independently, while a combined staining of these two markers such as colocalized staining, might provide a more robust proof of link between these markers, croton oil, and PMNs upregulation. Proteomic analysis also revealed that the MMP-8 protein was differentially expressed in croton oil-treated ears relative to acetone or naive and these results were confirmed using IHC. Thus, the proteomic analysis led us to the new discovery of the presence of MMP-8 in croton oil-induced inflammation which, to our knowledge, has not been reported previously.

The observation that topically applied acetone induces more than 2,000 proteins in mouse skin was unexpected and may help inform vehicle selection in future studies. The mechanism by which acetone achieves this response is also worthy of future study. Moreover, it was interesting to note that a series of proteins from the phosphatidyl inositol glycan (PIG) family, which are involved in the biosynthesis of glycosylphosphatidylinositols (GPIs) and their attachment to proteins to form GPI-anchored proteins (Kinoshita and Fujita, 2016), were induced by acetone in the mouse ear skin. To our knowledge, ours is the first report to show that the PIGN, PIGT, and PIGU proteins are increased in mouse skin exposed to acetone. Several mutations in the genes that code for these proteins have been associated with serious human diseases. *PIGN* mutations are associated with a rare condition called multiple congenital anomalies-hypotonia-seizures syndrome 1 (MCAHS1) (Khayat et al., 2016; Siavrienė et al., 2022). Mutations in *PIGU* impair the function of the GPI transamidase complex, and cause severe intellectual disability, epilepsy, and brain anomalies (Knaus et al., 2019). *PIGT* mutations are associated with intellectual disability syndrome (Kvarnung et al., 2013) and epilepsy (Ben Ayed et al., 2023). Moreover, at least one study has found that the PIGN protein is crucial in regulating mitotic integrity and serves to maintain chromosomal stability while preventing acute myeloid leukemic transformation/progression (Teye et al., 2017). Therefore, we propose a new purpose for acetone which is to experimentally induce PIGN, PIGT, and PIGU in tissues beyond the skin, which may be especially useful to researchers seeking to induce these proteins for mechanistic studies in cells and tissues, such as neurons and the brain. However, the extent to which the effect of acetone on cutaneous protein expression in mouse skin will be translatable to human cells and tissues, at the present time, remains unknown but is worthy of future study. Also, the thousands of other proteins induced by acetone in mouse ear skin relative to naïve tissue may confound the use of acetone to induce PIG proteins in human cells and tissues. Nonetheless, to our knowledge, an agent that induces PIGN protein levels in skin has not yet been reported in the literature.

There is at least one additional limitation to the work at hand that should be noted. First, compared to mouse skin, human skin has a thicker and multi-layered epidermis, sweat glands, sebaceous glands, and melanin. In contrast, the ear tissues of the Swiss Webster mice used here have a single to double layered epidermis and sebaceous glands but lack both sweat glands and melanin. More work is therefore required to determine the extent to which these observations will apply to human skin responses to croton oil. The work presented here was carried out strictly in mice and at a single time point (4 h) using a single exposure dose of croton oil; therefore, the data presented here should be extended in the future to include other time points and exposure doses of croton oil. Nonetheless, to our knowledge, the identification of MMP-8 in the tissue response to croton oil is novel and the effects of acetone on protein expression in mouse skin is worthy of note and of broad general interest to skin toxicologists.

CRediT authorship contribution statement

Ganming Mao: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Dalon Douglas:** Methodology, Formal analysis, Writing – review & editing. **Milankumar Prajapati:** Methodology, Formal analysis, Writing – review & editing. **Trishaal Janardhanam Raghavendra Rao:** Methodology. **Haiyan Zheng:** Methodology, Formal analysis, Writing – review & editing. **Caifeng Zhao:** Methodology, Formal analysis, Writing – review & editing. **Blase Billack:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision,

Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The teaching assistantships of G.M., T.J.R.R. and D.D. were funded by the Department of Pharmaceutical Sciences, St. John's University. This work was supported by funds from the National Institute of General Medical Sciences of the NIH under Award Number SC2 GM136612 (to BB) and Award Number R16 GM149512 (to BB) as well as by funds from the College of Pharmacy and Health Sciences, St. John's University, Queens, NY, USA. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The revisions to this manuscript were carried out by B.B. during his tenure as a U.S. Fulbright Scholar to Bulgaria at the Medical University of Plovdiv, Department of Pharmacology, Toxicology, and Pharmacotherapy (Faculty of Pharmacy) (Fulbright Bulgaria, Grant No. 23-11-06). The authors thank Xiuyi Liang, Richa Vartak and Prof. Ketankumar Patel of St. John's University and Dr. Rehab Hegazy of the National Research Centre (Giza, Egypt) for useful discussions and guidance regarding the present work. The authors dedicate this work to "Lenka," a beautiful young girl and friend of our lab who is struggling due to a homozygous mutation in *PIGN*. Special thanks are extended to Drs. S. Kumar and X. Cheng (St. John's University) for use of their digital imaging equipment for Western Blotting.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2024.100184.

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