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## Defining the timing of 25(OH)D rescue following nitrogen mustard exposure

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### Abstract

**Objective**—Mass exposure to alkylating agents such as nitrogen mustard (NM), whether accidental or intentional as during warfare, are known to cause systemic toxicity and severe blistering from cutaneous exposure. Thus, establishing the timing and appropriate dose of any potential drug designed to reverse or impede these toxicities is critical for wound repair and survival. Our previous data demonstrates that a single intraperitoneal injection of low-dose 25-hydroxyvitamin D3 (25(OH)D) given as early as 1 h following NM exposure is sufficient to rescue mice from pancytopenia and death. However, the duration of time following exposure where intervention is still effective as a countermeasure is unknown. In this study, we sought to assess the maximal time permissible following NM exposure where 25(OH)D still affords protection against NM-induced cutaneous injury. Additionally, we determined if a higher dose of 25(OH)D would be more efficacious at time interval where low dose 25(OH)D is no longer effective.

**Methods**—Low (5 ng) and high (50 ng) doses of 25(OH)D were administered intraperitoneally to mice following exposure to topical NM to assess wound resolution and survival. Mice were imaged and weighed daily to measure wound healing and to monitor systemic toxicity.

**Results**—We demonstrated that 5 ng 25(OH)D administered as early as 1 h and as late as 24 h post-NM exposure is able to achieve 100% recovery in mice. In contrast, intervention at and beyond 48 h of NM exposure failed to achieve full recovery and resulted in 60% death between days 6 and 12, demonstrating the critical nature of timely intervention with 25(OH)D at each respective dose. In order to circumvent the observed failure at >48 h exposure, we provided two consecutive doses of 5 ng or 50 ng of 25(OH)D at 48 h and 72 h post-NM exposure. Repeat dosing

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### Disclosure statement

The authors report no conflicts of interest.

with 25(OH)D at 48 h and beyond led to marked improvement of lesion size with 75% recovery from mortality.

**Conclusions**—The opportunity to use 25(OH)D as a medical countermeasure for NM-induced toxicity has a finite window for intervention. However, modifications such as repeat dosing can be an effective strategy to extend the intervention potential of 25(OH)D.

### Keywords

Nitrogen mustard; lesion; inflammation; wound; survival; 25(OH)D

## Introduction

Sulfur mustard (SM) is a DNA alkylating agent known to cause severe blistering and systemic toxicity from cutaneous exposure. Used as a chemical warfare agent in recent conflicts and during WWI, SM inflicted injury has drawn considerable attention and provided the impetus to identify countermeasures capable of mitigating SM effects and neutralizing the devastating cutaneous effects from its exposure<sup>1–3</sup>. Cutaneous exposure to SM triggers acute inflammatory lesions in the skin and in incidents of massive exposure, death<sup>4</sup>. Nitrogen mustard (NM), an analog of SM, has been used as a laboratory surrogate to develop animal models of cutaneous toxicity<sup>5,6</sup>. Although the DNA alkylating properties of NM have utility in a clinical setting for topical chemotherapy and treatment of cutaneous T cell lymphoma, such treatments have been associated with side effects that include cutaneous inflammation<sup>7</sup>. Thus, early intervention using an anti-inflammatory drug on patients receiving these therapies can potentially reduce inflammation-induced tissue damage and facilitate speedy recovery. Vitamin D (VD), an endocrine hormone responsible for maintenance of musculoskeletal health, is emerging as a powerful immunomodulatory drug responsible for suppressing inflammation<sup>8–10</sup>. We have recently demonstrated the efficacy of 25(OH)D to suppress early inflammation, mitigate tissue destruction and prevent systemic toxicity including death in animals exposed to NM<sup>9,11</sup>. Intervention with a single dose of 25(OH)D administered in a mouse model of NM-mediated cutaneous injury 1 h following exposure suppressed inflammatory mediators tumor necrosis factor- $\alpha$  and inducible nitric oxide synthase in the skin, promoted wound healing and ensured animal survival. Although 25(OH)D has a demonstrated critical role in reducing NM-induced skin inflammation, there is little evidence demonstrating the timing of the intervention in order to be effective in mitigating acute inflammation.

In this study, our goal was to evaluate the window of time allowed where intervention is still effective and assess the dose of 25(OH)D that needs to be administered at that time for recovery from NM-induced tissue destruction.

## Methods

### Mice

Six to eight-week-old pathogen-free female C57BL/6 J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Upon arrival, all mice were given a Vitamin D free laboratory diet purchased from TR Last Company (Cabot, PA), which was sustained through

the length of the study. All animal studies have been approved by the Case Western Reserve Institutional Animal Care and Use Committee. Mice groups were from 3 to 10 individuals depending on the group. All mice were caged individually for experiments in single-use disposable polystyrene cages in accordance with ABSL2 guidelines. The experiments conducted using this murine model of skin injury were part of an unblinded study.

### **Nitrogen mustard**

NM ( $\text{ClCH}_2\text{CH}_2$ ) $_2\text{NCH}_3 \times \text{HCL}$  was obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). NM (20  $\mu\text{L}$  of a 2.0% solution in 1.5% DMSO) was applied, at least 48 h after dorsal hair was removed with clippers and a depilating cream, to the dorsal surface of each mouse in an 8mm diameter area. Mice were anesthetized with an intraperitoneal (i.p.) injection of avertin for the procedure.

### **Vitamin D**

25(OH)D (Sigma Aldrich, St Louis, MO) was reconstituted in ethanol and then further diluted in mineral oil for use. 5 or 50 ng of 25(OH)D was injected i.p. at a single time point pre-exposure (-24 h), and at multiple time points post-NM exposure as a potential treatment for NM exposure, specifically 25(OH)D was injected 24 h before exposure and 1 h, 24 h, 48 h, 72 h and 96 h post-exposure.

### **Wound and weight measurements**

Wounds were measured from digital images taken daily with a Nikon Coolpix camera (Meville, NY) using Image J software. All mice were weighed daily using a Model CS 200 scale (Ohaus Corporation, Parsipanny, NJ).

### **Statistical analysis**

A two-sided unpaired Student's *t*-test was used to determine statistical significance. A log-rank test was used to determine statistical significance of survival curves. Data are presented as mean  $\pm$  S.E.M. and *p* values  $\leq .05$  were considered significant.

## **Results**

### **Intervention with 25(OH)D at early and late time points following topical NM exposure**

Gross images of mouse back show that topical application of NM on the dorsum of mice caused acute erythema that exacerbated to a cutaneous lesion as early as 24 h following exposure. If left untreated, the lesion progressively worsened and wound healing was delayed. Pretreatment with 5 ng 25(OH)D injected intraperitoneally 24 h prior to exposure had no protective effect on the lesion severity and wounds progressed in a manner similar to that of untreated controls. Treatment with a single low-dose of 25(OH)D (5 ng) administered intraperitoneally (i.p.) at 1 h or 24 h after NM exposure delayed wound induction and accelerated wound resolution associated with reduction in lesion area (Figure 1(A), circled, arrows). Compared to the 1 h or 24 h treatments, intervention with the same dose of 25(OH)D at 48 h, 72 h and 96 h produced little effect with wound lesions progressing in a time and fashion that was similar to untreated wounds (Figure 1(A)).

Quantitative analysis of lesion size validated the profile seen in the gross wound images. Intervention at both 1 h and 24 h post-NM exposure expedited reduction in lesion area compared to untreated wounds with a 66% ( $p = .002$ ) and 50% ( $p = .045$ ) decrease in wound area size in the mentioned treated groups, respectively, relative to untreated NM exposed mice measured at the end of the study (Day 20) (Figure 1(B), 5 mice per group). In contrast, 25(OH)D administered at 48 h was not effective in reducing lesion size (Figure 1(B)). In all other treatment groups including 24 h pretreatment and 72 h and 96 h treatment post-exposure, 25(OH)D failed to reduce lesion area and was comparable to untreated mice (Supplementary Figure 1). Given the devastating systemic effects of NM exposure, we assessed body weight loss in mice subjected to these conditions. NM exposure caused an observed biphasic weight loss with a sharp drop in weight at Day 1 post-NM exposure that was restored by nearly 50% by Day 5. A second drop in weight was observed at Day 5 that continued to reduce to 30% of their body weight by Day 10, which resulted in an unacceptable loss of body weight and ultimately sacrifice of animals that exhibited this degree of weight loss. After the first drop in weight on Day 1, mice treated with 25(OH)D at 1 h post-NM exposure exhibited a rapid return to baseline weight compared to control mice that was significantly different ( $p = .05$  on day 10) from the percentage weight loss of all other groups treated at and beyond 48 h (Figure 1(C), 5 mice per group).

All animals that failed to respond to pretreatment and treatment at time points 48 h were subject to a log-rank test on the Kaplan–Meier survival curve that indicated a significant difference between the successful and failed treatment groups ( $p = .04$ ) (Figure 1(D)). Taken together we observed that 5 ng of 25(OH)D was effective in facilitating recovery from weight loss and protecting mice from mortality with 100% recovery observed when 5 ng 25(OH)D was administered within 24 h of cutaneous NM exposure.

### Expanding the time limit for intervention

In an attempt to extend the window of time where animals exposed to cutaneous NM could still be rescued with Vitamin D past 24 h, mice were treated with a higher dose of 25(OH)D as well as two consecutive low and high doses of 25(OH)D in separate groups at later times following NM exposure. The late intervention and change in dose of 25(OH)D rescue modified wound development in a way that wounds progressed without any signs of recovery even with a high (50 ng) 25(OH)D dose injected at 48 h post exposure. In contrast, mice receiving two repeating doses of 25(OH)D (at 48 h and 72 h) showed improved wound resolution (Figure 2(A), circled, arrows). Measurement of wound sizes on Days 10 and 20 delineated some interesting trends – although 1 h intervention was significantly different from repeat dosing with 5 ng (Figure 2(B), Day 10, ( $p = .02$ ), there was no distinguishable difference in wound size between double dosing with either low (5 ng) and high (50 ng) doses of 25(OH)D on days 10 and 20 (Figure 2(B), (C), 10 mice per group for NM + VD(1 h), 8 mice per group for all other groups). Compared to a single 5 ng dose of 25(OH)D at 1 h post exposure, those that received a single 50 ng at 48 h showed robust wound progression when measured on Day 10 ( $p = .03$ ) and Day 20 ( $p < .0001$ ), Supplementary Figure 2 A,B). 72 h intervention group could not be evaluated on Day 10 as the met euthanasia criteria on day 6 (Supplementary Figure 2(A), (B)). Thus, the evolving trend suggested that late

intervention with a single low dose 25(OH)D was not as effective for reduction in wound size as was repeat dosing with either 5 ng or 50 ng 25(OH)D.

The systemic toxic effects of NM caused a severe drop in weight with animals losing 30% of their body weight. In contrast to 25(OH)D (1 h) treatment group that exhibited significant improvement in percent weight loss compared to untreated mice ( $p = .009$ ), treatment with a single dose of 50 ng 25(OH)D at 48 h underwent initial weight loss and although looked to recover body weight, their weights fluctuated with death on Day 12. Intervention at 72 h had the worst outcome with none surviving past Day 6 post-NM-exposure (Figure 2(D) line with asterisk,  $p = .009$ ). Corroborating with wound size, repeat dosing with 5 ng displayed significant weight loss relative to 25(OH)D at 1 h, ( $p = .02$ ) whereas repeat dosing with 50 ng did not; however, the overall effect of repeat dosing was more effective in resolving NM-induced skin wounds and weight loss than single dosing when intervened at and beyond 48 h postexposure [Figure 2(D), 8 mice for NM only, 10 mice for NM + VD(1 h), 3 mice per group for the two groups NM + VD 50 ng (48 h) and NM + VD 50 ng (72 h), 8 mice per group for the two groups NM + VD 5 ng (48 h, 72 h) and NM + VD 50 ng (48 h, 72 h)]. A log-rank test of the survival curve showed a significant difference amongst the different treatment groups with death occurring between days 5 and 13 ( $p < .0001$ ) (Figure 2(E)). Taken together we demonstrate that wounds show improved healing only when treated with not one but two consecutive doses of 25(OH)D administered at 48 and 72 h post NM exposure, at a time where a single dose is insufficient for tissue repair.

## Discussion

Mustard gas exposure affects various organs including lungs, bone marrow and skin leading to early and late manifestations of morbidity<sup>2,5,12,13</sup>. If left untreated, mustard induced skin injury is characterized by erythema and edema that progresses to vesication of the skin resulting in epidermal separation, vacuolization and epidermal necrosis<sup>14</sup>. In the interest of practical application of treating victims of mass exposure to chemical injury it is essential to determine the maximum length of time post-exposure that can lapse where treatment is still possible. In this study, we use a mouse model of NM-induced skin wound to evaluate the window of time within which 25(OH)D must be administered to accelerate wound healing and recovery from systemic toxicity. Furthermore, we assessed whether a higher dose of drug is more efficacious at a later time point when low dose therapy is no longer able to rescue mice from exacerbated inflammation and toxic effects of NM. Topical application of NM on an 8 mm area of the dorsum of mouse induced a wound as early as 1 day post-exposure. Without treatment, the lesion deepened and progressively worsened by day 10 post-exposure. The systemic toxic effects of NM reaches beyond local injury to cause cytopenia in blood, loss of intestinal barrier function, dilation of capillaries in the kidney, and bone marrow suppression<sup>9,15</sup>. Thus, the model is consistent with work previously reported by us and others that NM and SM induced skin vesication progressively deteriorates into a full thickness wound if continued inflammation is not suppressed by intervention with an anti-inflammatory drug<sup>9,16</sup>.

The immunomodulatory properties of vitamin D, a safe and FDA approved supplement, are being utilized in numerous immune pathologies and therefore an intense area of research.

Epidemiological surveys have demonstrated an inverse relation of 25(OH)D levels with severity of diseases such as inflammatory bowel disease (IBD), multiple sclerosis (MS), systemic lupus erythematosus as well as bacterial and viral infections<sup>17</sup>. These studies illuminate the wide scope of 25(OH)D effects including immunomodulation in multiple scenarios including but not limited to acute inflammation through suppression of prostaglandins, inhibition of transcriptional activation of NFκB signaling and inhibition in production of pro-inflammatory cytokines that exacerbate tissue damage<sup>18</sup>. Our previous success demonstrating the efficacy of 25(OH)D to suppress mustard-induced skin inflammation allowed us to test the critical timing of 25(OH)D intervention to diminish early skin inflammation from NM exposure. Compared to the remarkable effect of 25(OH)D administered 1 h post-exposure, we demonstrate that delaying drug administration by 24 h still achieves the same efficacy with delayed wound formation and accelerated wound recovery. While other groups demonstrate that SM causes delayed wound formation<sup>19</sup>, our data in NM mouse model reveals that 25(OH)D delivered at either 1 h or up to 24 h post-exposure was sufficient to delay the appearance of erythema and skin wound suggesting that 25(OH)D may inhibit uncontrolled activation of first responder cells such as neutrophils and macrophages that exacerbate inflammation at the wound bed. Although pretreatment with oral and topical calcitriol used as combination therapy<sup>20</sup> has proven efficacy in diminishing incidence of breast cancer and severity of IBD<sup>21</sup> in mouse models, in our hands pretreatment with 25(OH)D did not improve wound healing. This may be due to 25(OH)D arrest of infiltrating activated cells to the wound bed, a key inflammatory event that precedes the differentiation of reparative macrophages and is critical to wound healing<sup>22</sup>. We show that beyond 48 h a single low dose of 25(OH)D ceased to have any efficacy in skin wound recovery possibly because prolonged inflammation from uncontrolled activation of inflammatory cells at the site of NM injury led to irreparable tissue destruction. Studies in the autoimmune disease MS demonstrate the efficacy of cholecalciferol in mitigating hyper immune response when administered in repeated and increasing doses of D3<sup>23</sup>. Conforming to these studies we demonstrate that two consecutive doses of 25(OH)D administered at 48 h and 72 h, did indeed exhibit improved efficacy and was successful in diminishing wound area, reducing sharp drop in body weight of mice and preventing death from systemic toxicity and uncontrolled inflammation even after onset of robust inflammation.

## Conclusions

In this study, we establish that 25(OH)D-mediated recovery from NM-induced tissue destruction is effective only if a single low dose of 25(OH)D is administered within 24 h of exposure after which the same dose of 25(OH)D has limited efficacy. We also determined that beyond a certain window of time not one but repeated dose of 25(OH)D may be the most effective strategy to attain maximum efficacy at a time when the window for single dose has elapsed. These results have the potential to translate into practical applications such as a countermeasure for mass exposure to mustard gas, or to reduce the side effects of chemotherapy treatments. Increasing the window of time for intervention from chemical-induced injury translates into a more practical implementation of the countermeasure in the event of mass exposure. An additional desirable property of vitamin D is the relative safety profile with limited toxicity controlled by a series of hydroxylation steps in the liver and

kidney to convert vitamin D into its active form 1,25-dihydroxyvitamin d3 (1,25(OH)<sub>2</sub>D). As a countermeasure, vitamin D can be safely and efficiently administered orally to a large populations in the event of widespread exposure to alkylating agents even before establishing exposure status.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

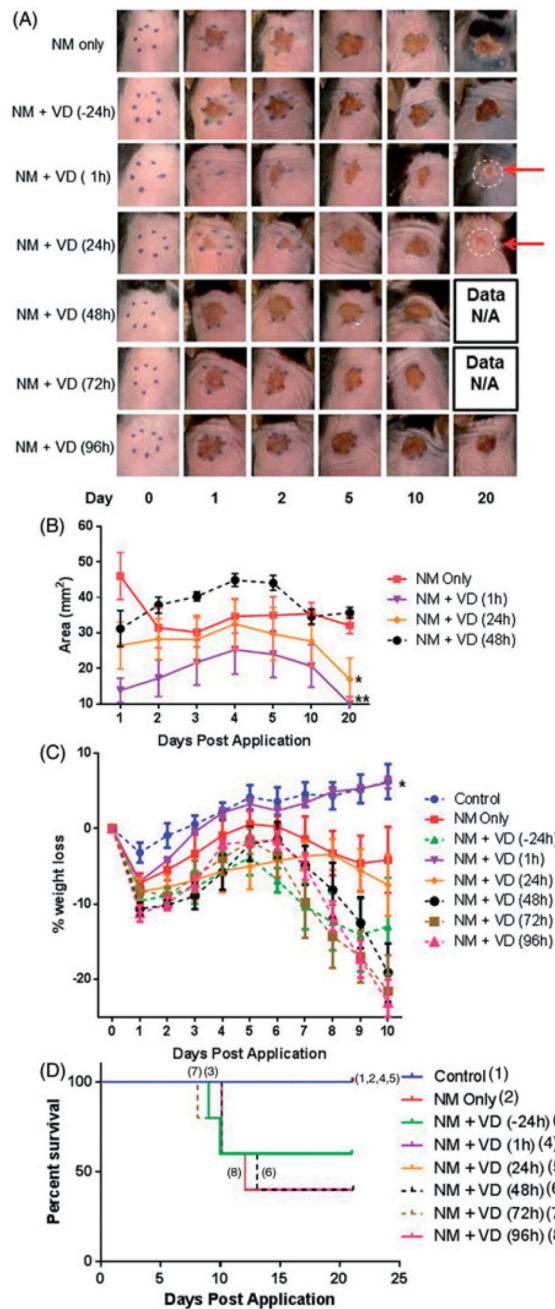
<b>VD, 25(OH)D<sub>3</sub></b>	Vitamin D
<b>NM</b>	nitrogen mustard

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**Figure 1.**

25(OH)D (VD) has a finite window of recovery from NM-mediated skin wounds. Mice were exposed to NM on their dorsal surface and 5 ng VD was injected intraperitoneally at various times as indicated. (A) Representative images of skin lesions on mice following NM exposure in the presence or absence of VD. Arrows indicate wound recovery. Data N/A indicates that the mice had met euthanasia criteria and were sacrificed. (B) Wound measurements of NM-exposed mice with and without treatment at indicated time points ( $p = .002$  on day 20 comparing NM only to VD at 1 h post exposure,  $p = .045$  on day 20 comparing NM to VD at 24 h post-exposure,  $n = 5$  mice per group). (C) Longitudinal

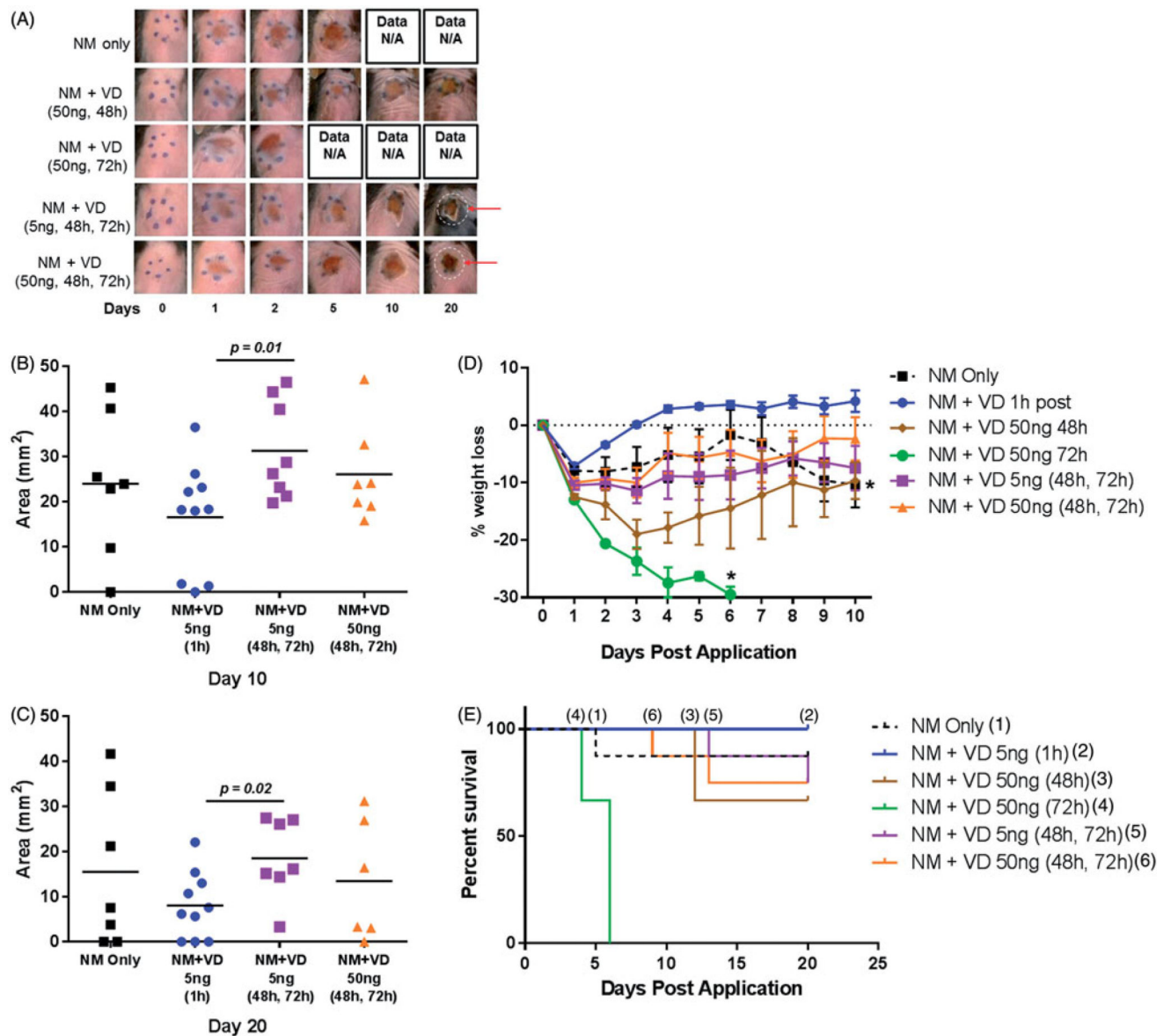
analysis of weight loss in mice in the treatment groups indicated ( $p = .05$  comparing NM only to VD at 1 h post-exposure on day 10) ( $n = 5$  mice per group). (D) Kaplan–Meier Curve demonstrating survival of NM mice at different times of treatment with VD, by log-rank test ( $p = .04$ ).

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**Figure 2.** Repeated doses of VD improves wound healing and promotes survival. Mice were exposed to topical application of NM on their dorsal surface and injected a single dose or 2 doses of 5 or 50 ng VD at indicated time points. (A) Representative images of skin lesions following NM exposure in the presence or absence of VD. Graphical evaluation of wound sizes of individual mice on (B) Day 10 and (C) Day 20 post NM exposure. (B, C)  $n = 10$  mice for NM + VD(1 h),  $n = 8$  mice for all other groups, (B)  $p = .01$  comparing NM + VD(1 h) with NM + VD 5 ng (48 h, 72 h), (C)  $p = .02$  comparing NM + VD(1 h) with NM + VD 5 ng (48 h, 72 h),  $p = n.s.$  for repeat doses of VD (5 ng) vs. repeat doses of VD (50 ng). (D) Longitudinal analysis of percent weight loss in mice treated with a single high dose of 50ng VD at 48 h and 50 ng VD at 72 h post-exposure compared with two consecutive doses of 5 ng VD or 50 ng VD at 48 h and 72 h. NM only and NM + VD(1h) is also included for reference comparison.  $p = .009$  comparing NM vs. NM + VD(1h),  $p = .009$  comparing NM

+ VD(1h) vs. NM + VD 50 ng (72 h).  $n = 8$  mice for NM,  $n = 10$  mice for NM + VD(1h),  $n = 3$  mice for NM + VD 50 ng (48 h) and for NM + VD 50 ng (72 h),  $n = 8$  mice for NM + VD 5 ng (48 h, 72 h), and for NM + VD 50 ng (48 h, 72 h). (E) Kaplan–Meier Curve demonstrating survival of NM exposed mice at different times and doses of treatment with VD. Log-rank test  $p < .0001$ .