**Original Article**

**Downregulation of miR-210 promotes sulfur mustard-induced skin wound healing**

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**Abstract:** Sulfur mustard (SM) is a chemical alkylating agent that causes severe vesicating skin injuries. However, the exact mechanisms have not been thoroughly elucidated, limiting the development and optimization of effective therapeutic interventions against SM. MicroRNAs (miRNAs) are small non-coding RNAs with ~22 nucleotide lengths, serving as important posttranscriptional gene regulators in cellular activities. Recently, miRNAs, especially miR-210, have been identified to be involved in skin wound healing. Thus, the present study aimed to determine the roles of miR-210 in SM-induced skin injuries in SKH-1 hairless mice. It was confirmed that SM significantly increased expression of miR-210 in skin from 3 days to 14 days post-exposure. miR-210 expressing cells were allocated to the epidermis by in situ hybridization. HIF1α, a key regulator of miR-210, was also increased in line with miR-210 expression. Injections of antago-miR-210 around the wound bed could efficiently lower miR-210 expression levels and promote wound healing. Moreover, antago-miR-210 application promoted keratinocytes migration to the vicinity of wounds. In conclusion, topical SM contamination in the skin could notably upregulate miR-210 expression, while downregulation of miR-210 could accelerate cutaneous wound healing, partially via promoting keratinocyte migration. Present results provide a therapeutic rationale of miR-210 on SM-caused skin injuries.

**Keywords:** Sulfur mustard, miR-210, keratinocytes migration, skin wound healing

**Introduction**

Sulfur mustard (SM; 2,2-dichlorodiethyl sulfide) has been classified as an extremely toxic chemical warfare agent since its first use in World War I, remaining a main threat even today for both military conflicts and terrorist activities [1]. Skin is the most important target tissue of SM, expressed as blistering [2]. After contact with the skin, SM induces erythema, blister formation, ulceration, and long-term effects, such as refractory wound healing [3]. Several clinical regimens have been tested for the development of antidotes to treat SM-exposed people, including antioxidants, ROS scavengers, anti-inflammatory drugs, and protease inhibitors. However, none of these have yielded a satisfying therapy [4-6]. A major limitation is the lack of understanding of specific and/or full mechanism(s) of SM-induced cutaneous toxicity, such as hallmark genes.

MicroRNAs (miRNAs), small non-coding RNAs, serve as important posttranscriptional gene regulators in controlling gene expression patterns of normal or stressed cells. They affect mRNA stability by causing mRNA degradation or translational repression, consequently controlling protein expression in cells. One miRNA may regulate a set of target genes. About 60% genes can be regulated by miRNAs [7]. Recently, miRNAs have been found to be involved in the regulation of numerous physiological and pathological processes of skin wound healing [8, 9]. It has been demonstrated that miRNAs act as both agonists and antagonists in the process of restoring barrier function of the skin. Expression tides of specific miRNAs during different phases could be associated with abnormal wound healing. Moreover, expression profiles of miRNAs in the serum of SM-exposed patients have been found to be significantly changed, in general. They have been considered as potential biomarkers for diagnosis and treatment of SM-exposed patients [10-12]. miR-210 is a hypoxia-induced miRNA in many cell types, including keratinocytes [13]. Accumula-
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ing evidence has indicated that miR-210 is involved in the regulation of mitochondrial metabolism, angiogenesis, DNA repair, and cell survival [14, 15]. It has been reported that miR-210 attenuates keratinocyte proliferation and impaired the closure of ischemic skin wounds in a murine model [16]. Moreover, a recent study identified that antago-miR-210 application to cultured keratinocytes could efficiently lower cellular miR-210 levels and block SM-induced cell differentiation in hypoxia conditions [17]. However, little information is available regarding the effects of miR-210 in SM-caused skin injuries.

The current study constructed a SM exposure SKH-1 hairless mouse model, successfully lowering miR-210 levels in the skin by injecting antago-miR-210. Present results, for the first time, suggest that SM contamination significantly upregulated expression of miR-210 in skin, while downregulation of miR-210 could accelerate cutaneous wound healing in SKH-1 hairless mice, partially via promoting keratinocyte migration. Present results offer new insight into the underlying mechanisms of SM-induced cutaneous toxicity, providing a therapeutic rationale of miR-210 on SM-caused skin injuries.

Material and methods

Animals

SKH-1 hairless mice (Crl: SKH1-hr) were purchased from Shanghai Public Health Clinical Center. Animals were maintained at a controlled temperature (22 ± 2°C) and a 12-hour light/dark period, with ad libitum access to water and food. About 6-week-old male SKH1-hr mice, 24 g-26 g in weight, were used. Animal experiments were carried out in strict accordance with recommendations in the Guidelines for the Care and Use of Laboratory Animals by the National Institutes of Health. Experiments were approved by the Animal Care and Use Committee of the Amy Medical University (Chongqing, China; Approval SYXC-2016-00115).

SM exposure

SM (96% purity) is a self-stored stock item in the department (AMU, Chongqing, China). Prior to SM exposure, mice were injected with 1% pentobarbital sodium (Merk, Germany) at a dose of 8 mL/kg. Next, mice were placed in a fume hood and a circular area of 0.5 cm² for SM exposure, marked using a seal on the dorsal-lumbar region of the animal centered on the body axis. Moreover, 2 μL of freshly-prepared SM suspension diluted with dichloromethane (V/V = 1:10) or solvent control was applied on the marked skin area. Four hours after SM exposure, exposed skin regions were gently wiped with sodium hypochlorite (0.8%) and saline for decontamination. Finally, the mice were housed, individually, in cages. Wounds were photographed by digital cameras every 3 to 4 days.

Quantitative real-time PCR for microRNA detection

At 1, 3, 8, and 14 days after SM exposure, skin wounds and nearby tissues were cut off. They were stored in TRIzol (Invitrogen, USA) for microRNA quantification. An electrical hand blender (Jinxin, Shanghai, China) was employed and set at 14000 rpm for 1 minute to mash skin tissue, due to its tenacity. To detect miR-210 levels, a commercial kit for miR-210 quantification by stem-loop RT-PCR was purchased (Ribbo Co, Guangzhou, China). RT-PCR and Real-time PCR were performed strictly according to manufacturer protocol. iQ5 (Bio-Rad, USA) and 2 × SYBR Green I (Promega, USA) were employed for real-time quantification. PCR results were normalized with 5S ribosome RNA as internal control, then expressed as relative expression levels and compared with corresponding control samples. ΔCt was calculated to quantitatively analyze results.

H&E and immunohistochemistry analysis

At 1, 3, 8, and 14 days after SM exposure, skin wounds and nearby tissues were cut off. They were fixed in 4% paraformaldehyde containing 0.1% DEPC for histology analysis. Subsequent preparation of paraffin blocks was taken as routine. Slice thickness was limited in 5 μm for H&E and immunohistochemistry (IHC). An IHC detection kit, based on SABC, was obtained from Boster (Wuhan, China). Primary antibodies for IHC detection were hypoxia inducible factor-1α (HIF-1α, Boster, Wuhan, China) and keratin 5 (K5, Santa Cruz, USA).

In situ hybridization

To analyze the distribution of miR-210, digoxigenin-labeled locked nucleic acid-modified de-
miR210 inhibition attenuates SM-caused skin injuries

Detection probes were pursued (612239-340, Exiqon, Copenhagen, Denmark). Paraffin blocks prepared in 2.4 were chosen for in situ hybridization (ISH) examination and slice thickness was limited to 7 μm. The detection process was strictly performed according to manufacturer protocol. AP-DIG antibody (Roche, Switzerland) was employed to engage with LNA miR-210 probe. The miR-210 signal was visualized in blue color by chromogenic agents BCIP/NBT (Boster, China).

miR-210 antagomir treatment

Chemically synthesized antagomir was used to disturb skin specific microRNAs [18, 19]. Prior to SM exposure, 16 μg miR-210 antagomir (Ribobio Co, Guangzhou, China) dissolved in 100 μL of PBS was injected into the peripheral zone around the marked area for SM exposure at three sites. The control group received equal amounts of scrambled antagomir. Progression of wound healing was digitally photographed at indicated time points. Area of the wound bed was calculated by digital planimetry using Image J software version 2.1.4.6 (NIH, Bethesda, MD). Wound tissues were collected for H&E and IHC staining at 8 days and 14 days after exposure.

Statistical analysis

Statistical analysis was conducted with t-test and one-way analysis of variance using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA). P-values < 0.05 indicate statistical significance. Tukey-Kramer post-hoc testing was applied if P < 0.05 for between-group comparisons.

Results

SM increased miR-210 expression in skin

In accord with previous observations [20, 21], the present SM-exposed SKH1-hr mouse model displayed hyaline degeneration, erythema, and significant crusts, accompanied with tissue fluid exudation. In mice with thermal burns of full-thickness skin, the shedding of eschar and wound healing are usually completed around 10 days and 3 weeks after the injury, respectively. In the current mouse model, the whole healing process was postponed, with eschar shedding occurring 17-20 days after the injury. An ulcer in the place of crusts could be seen (Figure 1A and 1B). Under the microscope, the epidermis of SM-wounded skin was thicker than that of control (Figure 1C). As shown in Figure 1D, expression of miR-210 in the skin was increased nearly 3-fold, compared to the control group, 3 days post-SM exposure. It maintained at this high level for up to 14 days post-SM exposure. Moreover, expression of HIF1α, a key up-regulator of miR-210 [22], was dramatically induced in epidermal cells 1 day and 3 days post-SM exposure (Figure 1E and 1F). Current results suggest that SM caused typical skin injuries and induced expression of miR-210.

miR-210 expressed in epidermis in skin after SM exposure

Performing ISH using the LNA-microRNA probe, the current study detected miR-210 expression in cells scattered in the skin at 1, 3, and 8 days post-SM exposure. As shown in Figure 2A-C, SM significantly induced expression of miR-210 in epidermal cells. At 3 days after SM insult, many cells in the epidermal layer expressed miR-210. At 8 days, with the amplification of epidermal cells, strong miR-210 expression expanded to many cell layers. Additionally, the positive signal of miR-210 was mainly located in the spinous and granular layers of epidermis. Results indicate that induction of miR-210 expression in SM-exposed skin was mainly distributed in epidermal cells.

Anti-miR-210 accelerated wound healing of SM-injured skin

As shown in Figure 3A, antago-miR-210 injections significantly blocked SM-induced miR-210 expression at observed time points. To evaluate the effects of antago-miR-210 on wound healing of SM-caused skin injuries, the process of wound healing was digitally photographed at 5, 8, 11, 14, 18, and 21 days post-SM exposure. Within the first 5 days after SM exposure, antago-miR-210 treatment did not show obvious effects on the gross pathological signs of the wounds (Figure 3B and 3C). At 8 days post-SM exposure, the eschar area was significantly shrunken in the antago-miR-210 injection group, accompanied with more newly re-epithelialized granulation tissues around. The bright red color indicated abundant capillaries (Figure 3B and 3C). At 11 days post-SM exposure, further contractions of the wound
miR210 inhibition attenuates SM-caused skin injuries

Figure 1. SM induced expression of miR-210 in skin. SM was applied to the dorsal surface of each mouse, as described in the Materials and methods section. A. Representative images of skin wounds post-SM exposure. The process of wound healing was digitally photographed at 1 d, 3 d, 5 d, 8 d, 11 d, 14 d, 17 d and 23 d post-wounding. B. The residual areas of SM wounded skin were outlined and analyzed using image J. software. Data shown as mean ± SD; n = 6; **, P < 0.01; compared to SM exposure 1 d group. C. H&E staining was performed to analyze injured skin at 1 d and 3 d post-SM exposure. D. Real-time PCR quantification of miR-210 expression in skin at 1 d, 3 d, 8 d, and 14 d after SM exposure. E. IHC of HIF-1α was performed in the injured skin at 1 d and 3 d post-SM exposure. Red arrows indicate the positive signal of HIF-1α. F. By counting the number of HIF1-α positive cells and the total cells, the percentage of HIF1-α positive cells was calculated. Data shown as mean ± SD; n = 6; **, compared to the control group, P < 0.01. e, epidermis; d, dermis.

Bed led to a much smaller residual wound area in the antago-miR-210 injection group, compared to the control group. Significant granulation tissue formation and re-epithelialization could be seen around the crust after antago-miR-210 intervention (Figure 3B and 3C). At 14 days post-SM exposure, most crusts were shading off and showed a residual ulcer in the antago-miR-210 injection group. However, crusts in the antago-control injection group were still covered the wound bed, of which the shrinking was weak (Figure 3B and 3C). Current results suggest that antago-miR-210 markedly inhibited SM-induced miR-210 expression, thereby promoting skin wound healing.

Anti-miR-210 accelerated wound healing of SM-injured skin by promoting keratinocyte migration

Migration of keratinocytes is one of the essential biological processes for epidermal repair [23]. Therefore, this study aimed to determine the roles of miR-210 in keratinocyte migration. H&E and IHC staining were performed to analyze histology changes of the skin. K5 was used as the indicator of keratinocytes, as reported before [24]. Since antago-miR-210 had a significant effect on skin wound healing at 14 days after SM exposure, samples at this time point were chosen for histopathology analysis. Under the microscope, the wound tissue was designated as SM-contaminated area (SM) and non-contaminated skin (clean) for the purposes of easy description. The epidermis in the SM-exposed area was abnormal with little cell nucleus staining. The morphology of clean area was in accord with normal skin (Figure 4A). Moreover, this study observed many cells in the epidermis of the clean area migrated to SM-injured skin, forming a thin and clear migrating line underneath the crust. These were proven to be undifferentiated keratinocytes by K5 staining (Figure 4A). Comparing their migrating distance, it was found that the keratinocyte migrating line in the antago-miR-210-injected group was nearly two-fold longer than that in the antago-control-injected group (Figure 4B). Current results raise the possibility that antago-miR-210 accelerated skin healing by enhancing keratinocyte migration.

Discussion

SM-caused skin injuries currently lack effective therapies, though some benefits have been
miR210 inhibition attenuates SM-caused skin injuries

obtained with decontamination and supportive treatment, if applied in a timely manner [25, 26]. The major limitation is that the underlying mechanisms of SM injury have not been well-understood [26]. The present study demonstrated that SM increased expression of miR-210 in the skin and targeting SM-induced miR-210 expression significantly promoted skin wound healing through enhancing keratinocyte migration, at least partially. To the best of our knowledge, this is the first report concerning the potential therapeutic effects of miR-210 against SM-induced skin injuries in vivo.

MicroRNAs regulate gene expression by directly binding to the 3′untranslated region (3′UTR) of specific target mRNA(s) with incomplete sequence pairing, leading to the degradation and/or hindering of the translation of mRNA. This leads to decreased protein levels of target genes [27]. Many studies have shown that skin wound healing is controlled by miRNAs via regu-

Figure 2. miR-210 expressed in epidermis in skin after SM exposure. A. At 1 d, 3 d, or 8 d post-SM exposure, ISH were performed to locate miR-210 in injured skin. Random fields of vision were captured for analysis. Red arrows indicate a positive signal of miR-210. B. By counting the number of miR-210 positive cells and the total cells, the percentage of miR-210 positive cells was calculated. C. H&E staining was performed to analyze the injured skin at 1 d, 3 d, and 8 d post-SM exposure. Data shown as mean ± SD; n = 6; **, compared to the control group, P < 0.01. e, epidermis; d, dermis.
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Figure 3. Anti-miR-210 accelerated SM-caused skin wound healing. A. Expression of miR210 analyzed by qPCR. B. Representative images of skin wounds post SM exposure. The process of wound healing was digitally photographed at 5 d, 8 d, 11 d, 14 d, 18 d, and 21 d post-wounding in both control and antago-miR-210-treated groups. C. The residual areas of SM wounded skin were outlined and analyzed using image J software. Data shown as mean ± SD; n = 6; *, P < 0.05; **, P < 0.01; compared to the Antago-control injected group.

Expression of various cellular processes, including cell cycle, proliferation, senescence, and apoptosis. Yumin Zhu et al. [28] found that overexpression of miR-29b reduces collagen biosynthesis by inhibiting heat shock protein 47 during the healing of excisional or burned skin wounds in mice. Longlong Yang et al. [29] reported that miR-155 accelerates wound healing, probably mediated by accelerating keratinocyte migration via upregulated MMP-2 levels, according to a full thickness skin wound model of SD rats. Dongqing Li et al. [30] showed that miRNA-31 promotes skin wound healing by enhancing keratinocyte proliferation and migration in surgical wounds of abdominal skin. Previous results also indicate that miR-198 may be a key regulator in keratinocyte growth [31]. Reportedly, miR-20a and miR-21 were downregulated in SM-exposed skin lesions, as well as miR-92a and miR-20a in the serum of patients with mustard lung, compared to the control group [10, 11]. Out of 667 investigated miRNAs, 66 showed a significant change of expression in endothelial cells upon incubation with SM [12]. These significant changes of miRNA expression profiles in SM-exposed patients suggest the potential use of miRNAs for diagnosis and treatment of casualties. In 2016, Janina Deppe et al. found that upregulation of miR-210 affected the growth and differentiation of cultured keratinocytes after exposure to SM in hypoxia, but not in normoxia [17]. However, the present in vivo study demonstrated the upregulation of miR-210 in SM-exposed mice under normoxia conditions. Possible explanations could be (1) Different responses between in vitro and in vivo experiment models; and (2) Dysfunction of capillary circulation caused insufficient supply of oxygen and nutrients at the wound site. Moreover, the current study found that downregulation of miR-210 accelerated SM-caused skin wound healing. Present results complemented previous work on the roles of miR-210 in SM-induced skin injuries, perhaps opening new avenues to therapeutic strategies for skin wound healing caused by SM.

Furthermore, potential mechanisms of the beneficial effects of antago-miR-210 on SM-induced skin wound healing were investigated. It has been demonstrated that migration of keratinocytes is a crucial cellular event during skin wound healing and essential for re-epithelialization [32]. Previous studies have indicated that keratinocyte migration plays an important role in the effects of miR-31 and miR-155 on skin wound healing [29, 30]. Recently, Biswas et al. found that hypoxia-induced upregulation of miR-210 in keratinocytes resulted in diminished cell proliferation and limited re-epithelialization, according to a murine model of ischemic wounds [16]. The current study, for the first time, found that miR-210 antagonimir treatment markedly increased keratinocyte migration, indicating that downregulation of miR-210 could promote wound healing mainly through accelerated keratinocyte migration in SM-tra-
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Figure 4. Anti-miR-210 promoted keratinocytes migration. After 14 d of SM exposure, skin from the antago-miR-210 treated group and the control group was collected for histology analysis. The wound tissue was designated as SM contaminated area (SM) or non-contaminated skin (clean) for the purpose of easy description. A. H&E staining and IHC of K5 were performed. Random fields of vision were captured for analysis. Dotted lines in SM of antago-miR-210-treated group or control indicate specific keratinocytes which were migrating to injured skin. B. The migrating distance of keratinocytes was calculated from the beginning of SM injured skin until the area where keratinocytes disappeared by image J software. Data shown as mean ± SD; n = 6; ***, P < 0.01; compared to the antago-control injected group.

In general, present findings, for the first time, provide direct in vivo evidence that downregulation of SM-induced miR-210 expression promotes skin wound healing, partially through increasing keratinocyte migration in SKH-1 hairless mice. Present results open a new avenue of research regarding the potential mechanisms of SM-induced skin injuries, complementing the therapeutic effects of miRNAs on skin wound healing.

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Disclosure of conflict of interest

None.

Abbreviations

HIF-1α, hypoxia inducible factor-1α; IHC, immunohistochemistry; ISH, in situ hybridization; K5, Keratin 5; miRNAs, MicroRNAs; SM, Sulfur mustard.

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