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Effects of the biocide methylisothiazolinone on *Xenopus laevis* wound healing and tail regeneration

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ABSTRACT

The South African clawed frog, *Xenopus laevis*, has a strong history as a suitable model for environmental studies. Its embryos and transparent tadpoles are highly sensitive to the environment and their developmental processes are well described. It is also amenable for molecular studies. These characteristics enable its use for rapid identification and understanding of exposure-induced defects. To investigate the consequences of chemical exposure on aquatic animals, *Xenopus laevis* embryos and tadpoles were exposed to the biocide, methylisothiazolinone (MIT). Frog tadpoles exposed to MIT following tail amputation lost their natural regenerative ability. This inhibition of regeneration led to a failure to regrow tissues including the spinal cord, muscle, and notochord. This MIT-dependent regenerative defect is due to a failure to close the amputation wound. A wound healing assay revealed that while untreated embryos close their wounds within one day after injury, MIT-treated animals maintained open wounds that did not reduce in size and caused lethality. Concomitant exposure of MIT with chemicals containing thiol groups such as glutathione and N-acetyl cysteine restored normal wound healing and regeneration responses in tadpoles. Together these results indicate that exposure to MIT impairs developmental wound repair and tissue regeneration in *Xenopus laevis*. Thus, this study reveals new aspects of MIT activity and demonstrates that *Xenopus laevis* is a well-suited model for facilitating future research into chemical exposure effects on injury responses.

1. Introduction

The anuran frog *Xenopus laevis* is a molecularly tractable model widely used for biomedical research, including developmental biology, cell biology, biochemistry, neurobiology, and regeneration (Chen and Lin, 2014; Gurdon and Hopwood, 2000; Khokha, 2012). Moreover, the development of *Xenopus laevis* embryos is external, rapid, and well understood, facilitating quick identification of cellular and molecular pathways (Heasman, 2006). *Xenopus laevis* enables multi-level studies from the gene to cellular to tissue to organismal levels (Grant et al., 2015; LaBonne and Zorn, 2015; Wheeler and Liu, 2012). It is also a highly useful, though currently under-utilized, model for environmental studies (Berg et al., 2009; Dumpert, 1987; Mouche et al., 2011).

Xenopus laevis has a long history as a model for environmental exposure and related studies. Frog embryos are cultured in normal petri dishes and develop into tadpoles within five days (Sive et al., 2000). Moreover, tadpoles are transparent, making it easy to identify potential tissue and developmental defects. The Frog Embryo Teratogenesis Assay Xenopus (FETAX) assay is an established method to assess developmental toxicity of chemicals (Dumont et al., 1983). It is reliable and gives similar *in vivo* results as mammalian studies (Fort et al., 2000; Leconte and Mouche, 2012). In addition, studies of chemicals effects in *Xenopus laevis* (including endocrine disrupting compounds, hormones, and environmental pollutants) have contributed to the understanding of their toxicological impact (Bevan et al., 2002; Collier et al., 2008; Gao et al., 2016; Isidori et al., 2016; Kim et al., 2013; Levy et al., 2004; Li et al., 2016; Tompsett et al., 2013; Zaya et al., 2011).

Aquatic toxicology studies using *Xenopus laevis* have focused on identifying and understanding effects on animal development, reproductive health, and environment (Güngördü et al., 2016; Hayes et al., 2010; Haywood et al., 2004; Slaby et al., 2016). However, there may be additional chemical exposure effects which may not be identified readily but which could be of important consequence for survivability. For example, the tadpole tail is a key

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appendage for survival. It is not only needed for swimming but may also aid in escape from predator (Hoff and Wassersug, 2000; Seckendorff Hoff and Wassersug, 1986). An inability to heal wounds in the tail can lead to tadpole death (Ho and Whitman, 2008). Thus, ineffective responses to injuries may negatively impact the ability of an animal not only to heal but also overall health, and the ability to escape from predators.

The *Xenopus laevis* tadpole tail is a complex structure with spinal cord, notochord, muscle, epidermis, and vasculature (Beck and Slack, 1999). The tail appendage has similar major tissues and structures as a human limb. But in contrast to human limbs, the frog larval tail has high regenerative ability. It can regenerate lost tail structures. The tail of a developmental stage 40 tadpole (Fig. 1A, approximately five days after fertilization) can be amputated (Fig. 1B) to assay for regeneration. Following injury, *Xenopus laevis* tail regeneration is comprised of three major steps: wound healing, initiation of regeneration marked by the establishment of the "regeneration bud", and tail outgrowth and patterning (Tseng and Levin, 2008). Wound healing occurs in the first 0–12 h after amputation (hpa) (Ho and Whitman, 2008). After successful wound healing, an outgrowth called the "regeneration bud" is observed at the injury site and contains the tissue-specific stem cells needed for restoring the tail (Fig. 1C,D) (Slack et al., 2004). After 24 h, growth pathways that drive appendage extension, innervation, and patterning, are activated (Chen and Lin, 2014; Tseng and Levin, 2008). By seven days, the tail is successfully restored (Fig. 1E Stage 46–swimming tadpole stage) (Tseng et al., 2007).

Methylisothiazolinone (commonly known as MIT) is a biocide that acts as a broad-spectrum antimicrobial agent and is used to control the growth of bacteria, fungi, algae, slime, and mold (Collier et al., 1990). Found in numerous household and personal products (including wall paint, air conditioners, shampoos, detergents, cosmetics, lotions, and baby wipes), MIT is used to extend product shelf life. In industrial applications, such as water cooling systems and fuel storage tanks, it is used at higher concentrations to block microbial growth. The noted surge in contact dermatitis (skin reactions) cases has been attributed in part to MIT exposure (De Groot and Herxheimer, 1989; Monsálvez et al., 2011). Similarly, occupational exposure to MIT at significantly higher doses than consumer products is known to cause severe burns (Gruvberger and Bruze, 1998). MIT has also been reported to be toxic to aquatic animals including freshwater fish, daphnia, and *Xenopus laevis* tadpoles (EPA, 1998; Spawn and Aizenman, 2012). However, the cellular effects and mechanisms of MIT function remain unclear.

MIT contains an active thiol moiety that reacts with molecules which contain SH residues (Collier et al., 1990). As MIT exposure may be a potential health issue for aquatic animals and humans, there are few studies on the mechanisms of MIT action in animal models. To date, several reports have examined MIT effects in cultured cells. It can act as a neurotoxin, inhibiting neural outgrowth at lower concentrations while inducing apoptosis at higher ones (Du et al., 2002). MIT also disrupts association of Src kinase with FAK in cultured neurons (He et al., 2006). In addition, MIT exposure is known to induce cell death in human keratinocytes *in vitro* (Ettorre et al., 2003). However, the *in vivo* effects of MIT action on animals remain largely unexamined. A report indicated that at sublethal concentrations, MIT-exposed *Xenopus laevis* tadpoles showed defective neural behavior (Spawn and Aizenman, 2012). In this study, we demonstrate that *Xenopus laevis* is a suitable model for studying the consequences of chemical exposure on animal injury responses.

The aim of this study is to investigate the effects of MIT on *Xenopus laevis* tadpole tail regeneration and wound healing. To assess the consequence on regeneration, we examined the tail regeneration ability of tadpoles exposed to MIT as compared to untreated animals. To determine the regenerative defects induced by MIT exposure, we used molecular markers to examine the cellular changes on specific tail tissue types. We then determined the temporal requirement for MIT to inhibit tail regeneration. We also assessed whether MIT exposure causes wound healing defects. Lastly, we hypothesized that by using antioxidants glutathione (GSH) or N-acetyl Cysteine (NAC) to provide additional thiol groups to interact with MIT, these chemicals would protect thiol groups in critical cellular proteins, rescuing these substrates from MIT modifications. Thus we investigated whether presence of antioxidants can prevent MIT-induced regeneration defects.

2. Materials and methods

2.1. Animal care and tail regeneration assay

Adult *Xenopus laevis* frogs were purchased from Nasco (Janesville, WI). *Xenopus laevis* were grown via approved protocols and guidelines (UNLV Institutional Animal Care and Use Committee). Embryos were obtained via *in vitro* fertilization and were raised in 0.1X Marc's Modified Ringer (MMR; 0.1 M NaCl, 2.0 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.8) medium (Sive et al., 2000). Tail regeneration assay was performed as described (Tseng et al., 2007). In brief, tadpoles at stages 40/41 (Nieuwkoop and Faber, 1994) were anaesthetized with 0.01% tricaine methanesulfonate. Tails were amputated midway between the cloaca and tip using a scalpel blade. After surgery, tadpoles were transferred into 0.1X MMR, allowed to recover, and then cultured in 0.1X MMR (with or without reagent) at 22 °C for seven days and scored for tail regeneration. To quantify and compare regeneration in groups of tadpoles treated with different dosages and/or reagents, we assigned each tail regenerate into one of four phenotype categories (full, good, weak, none) as described previously (Tseng et al., 2010). Full regeneration represents complete reconstitution of the tail with all major components (spinal cord, notochord, fin, and muscle). Good regeneration represents a tail with major components but missing some fin tissue. Weak regeneration represents a tail stump with visible but very little tissue regrowth. None indicates that no regenerate tissue was observed. Each experiment was performed three times with batches of tadpoles ($n > 30$ for each condition) resulting from fertilizations of different females.

2.2. Reagents

Chemicals used include methylisothiazolinone (Sigma Aldrich, St. Louis, MO), glutathione (TCI America, Portland, OR), and N-acetyl cysteine (Amresco, Solon, OH). Methlyisothiazolinone, glutathione, and N-acetyl cysteine were dissolved in deionized water to make stock solutions, which were stored at −20 °C. Specific treatment concentrations were made by diluting the stock solutions with 0.1X MMR. As this study focused on tissue regeneration and wound healing, chemicals were used at concentrations that enabled normal embryo and tadpole development based on external morphology. Concentrations of methylisothiazolinone used include 50 μM, 75 μM, and 100 μM. Embryos or tadpoles were transferred to medium containing chemicals after experimental surgery and brief recovery time. Solutions were not changed during the length of the seven-day treatment. The effect of regeneration inhibition was similar when MIT was changed daily ($n = 40$ per condition for three replicates, $p > 0.2$).

2.3. Immunohistochemistry

Tadpoles were fixed in MEMFA (100 mM MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO4, and 3.7% formaldehyde). Antibody stainings were performed according to (Sive et al., 2000). Primary antibodies used include: 12/101 (1:5; muscle), Xen1 (1:50; neural

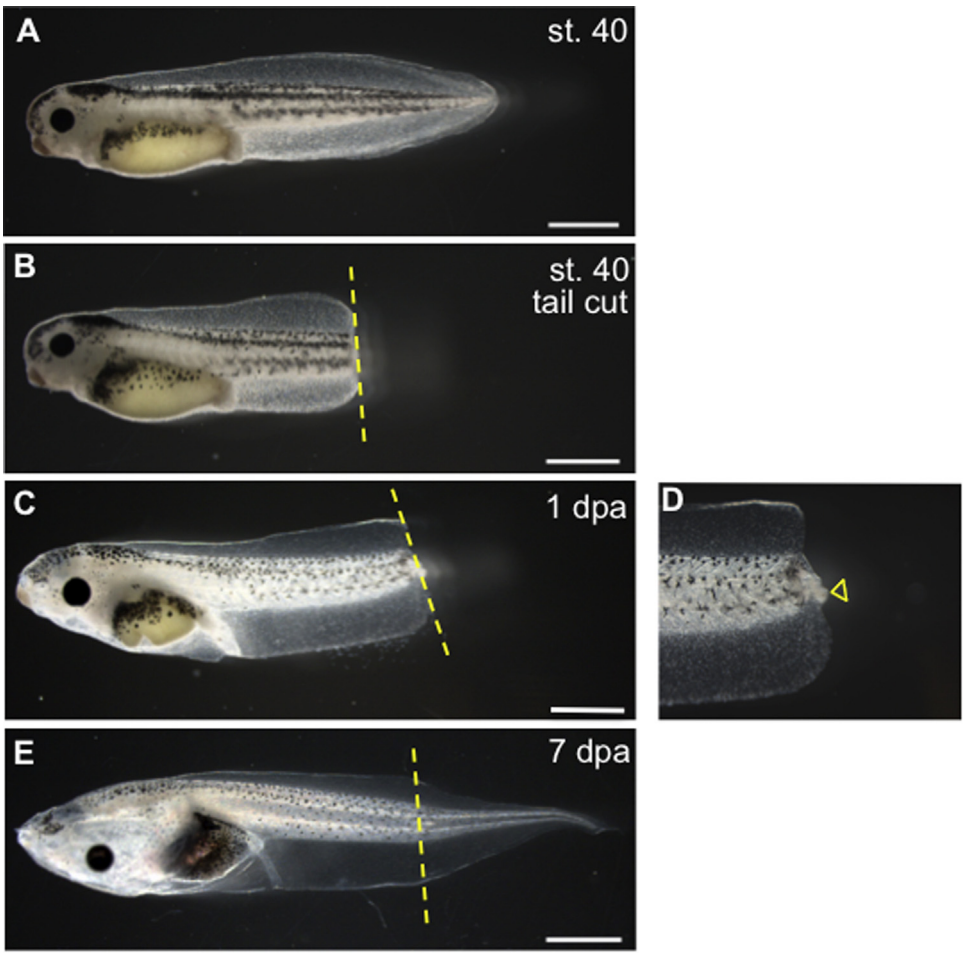

Fig. 1. Tail Regeneration in *Xenopus laevis*.
(**A**) A developmental Stage 40 tadpole. (**B**) A Stage 40 tadpole with tail amputated midway from the tip of the tail to the cloaca. (**C**) At one day post amputation (dpa), a regeneration bud is formed as shown in (**D**, open arrow). (**E**) By seven days, a fully formed tail is regenerated. Dashed lines show the plane of amputation. Scale bar = 2 mm

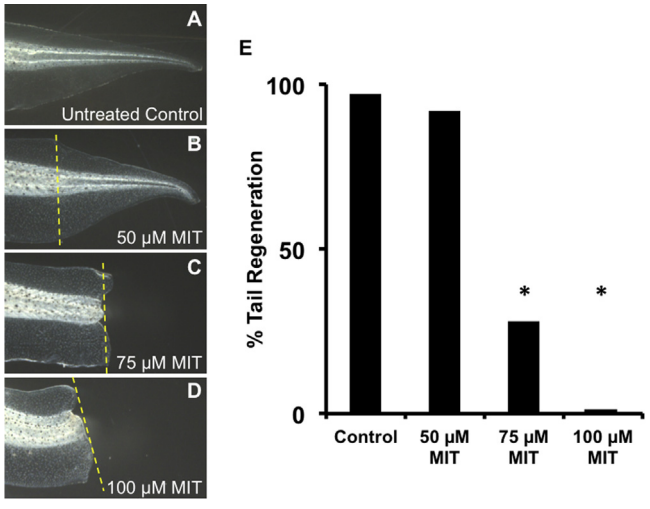

Fig. 2. Effects of MIT on Regeneration.
(**A-B**) Untreated tadpoles and tadpoles treated with 50 μM MIT regenerate tails. (**C-D**) Tadpoles treated with 75 μM MIT and 100 μM MIT fail to regenerate tails. (**E**) Quantification of tail regeneration showing the effect of MIT on tail regeneration. Dashed lines represent the plane of amputation. Results were compared using Kruskal-Wallis test. * denotes $p < 0.01$ as compared to control. N = 30 for each treatment, which was replicated three times.

tissues), and MZ15 (1:50; notochord) from Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA), and anti-acetylated α-tubulin (1:500; axonal patterning) from Sigma-Aldrich (St. Louis, MO). Alexa Fluor secondary antibodies from Life Technologies (Carlsbad, CA) were used at 1:500 dilution. DAPI (4′,6-diamidino-2-phenylindole) (Life Technologies), a commonly used

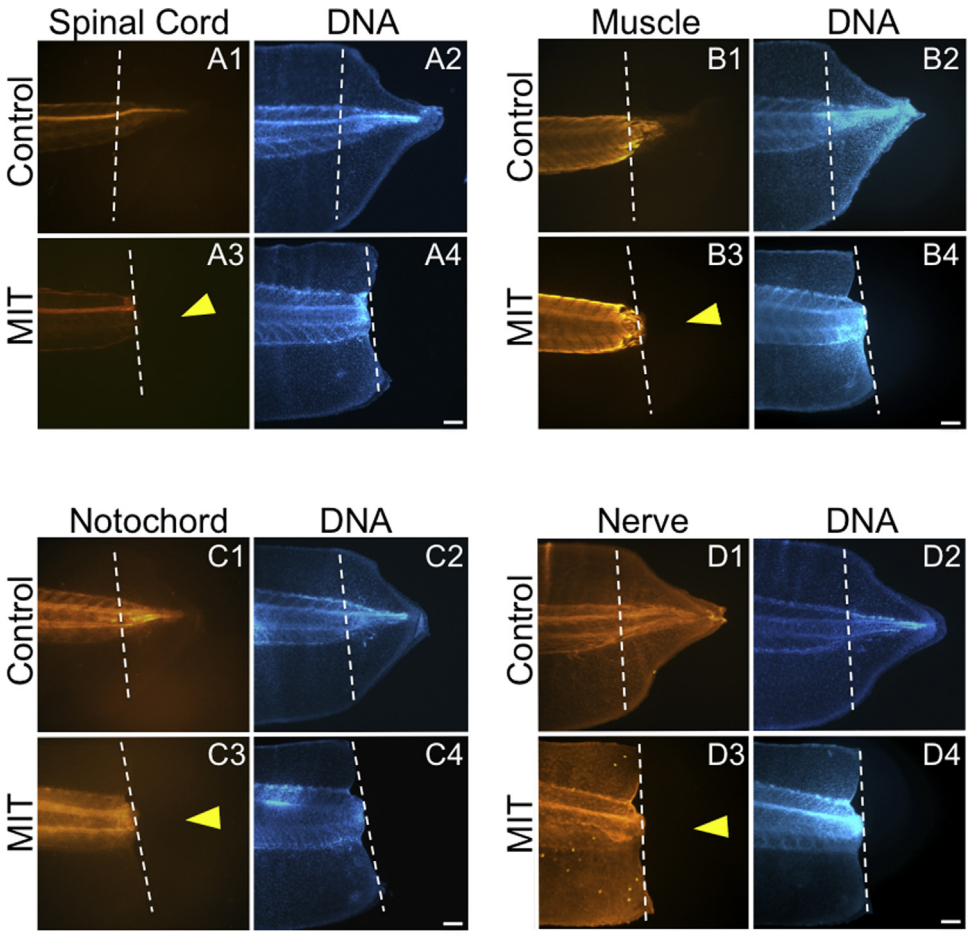

Fig. 3. Effects of MIT on Tissue Regrowth.
Tadpole tails were stained using: (**A**) spinal cord marker, Xen1, (**B**) muscle marker, 12/101, (**C**) notochord marker, MZ15 and (**D**) nerve marker, anti-acetylated tubulin. DAPI was used to visualize DNA. Compared to untreated tadpoles (**A1-A2**), tadpoles treated with MIT (**A3-A4**) lack spinal cord regrowth after amputation. Muscles also begin to degenerate in MIT-treated tadpoles (**B3**) compared to untreated tadpoles (**B1**). Notochord growth is also inhibited in MIT-treated tadpoles (**C3**) as compared to the notochords (**C1**) of untreated controls. Nerve regrowth was also absent after amputation in tadpoles treated with MIT (**D3**), as compared to untreated tadpoles (**D1**). Dashed lines represent amputation planes, yellow arrows represent missing tissues. Scale bar = 2 mm.

fluorescent DNA stain that binds strongly to A-T regions, was used as a nuclear stain for cells in tail regenerates. Each experiment was performed three times with tadpoles ($n > 10$ for each antibody) resulting from fertilizations of different females. Images were acquired using a Zeiss Axio Imager 2 fluorescent microscope.

2.4. Temporal treatment

To assess when exposure of MIT is most critical for disruption of tail regeneration, tadpoles were exposed to 75 μM MIT for different durations of time. To exclude the possibility that MIT effects perdure after its removal, temporal experiments were performed where MIT was added at specific time points (12 and 24 hpa) after tail amputation. Each experiment was performed three times with batches of tadpoles ($n > 30$ for each condition) resulting from fertilizations of different females.

2.5. Wound healing assay

The tail regeneration assay can be used to detect deficiencies in wound closure. However, it is within the context of regeneration. To directly test for impairment in wound closure, a classical *Xenopus laevis* embryo wound healing assay (Rajnicek et al., 1988; Yoshii et al., 2005) was used. Frog embryos are able to heal after bisection and both the head and tail halves continue to develop independently (Rajnicek et al., 1988). Embryos at Stage 25 were de-vitellinized and allowed to heal superficial nicks that may have occurred during devitellinization. Once healed, embryos were transversely bisected into equal halves using a scalpel blade. Embryos halves were transferred to control plates ($n = 10$) and MIT containing plates ($n = 10$ per treatment). MIT concentrations for treatment plates included 50 μM and 75 μM. Experiment was replicated three times with embryos from three different fertilizations. Images were acquired using a Zeiss V20 Stereomicroscope to track wound areas at indicated time points post bisection. Wound measurements were obtained using Zen software (Zeiss). For each image, the total surface area of the initial wound and individual wound areas at specific time points were outlined and total pixel area measurement obtained for each image. To determine the percentage of wound healed, the wound area at a specific timepoint was subtracted from the total initial wound area at the injury site; this number was then divided by the total initial area of the bisection site.

2.6. Antioxidant treatment

MIT contains an active sulfur moiety that reacts with molecules containing sulfhydryl (SH) groups, such as those found in cysteine residues (Collier et al., 1990). MIT also reacts with protein thiols *in vitro* (Frerot et al., 2013). These observations indicate that

exposure to MIT may cause covalent modifications that alter protein functions. The antioxidants N-acetyl cysteine and glutathione were used at 75 μM, a 1:1 molar ratio to MIT. Each chemical was added separately to the experimental plate. Each experiment was performed at least three times with tadpoles provided by three different fertilizations from different females and males. Each replicate had n ≥ 30 for control and each treatment plate.

2.7. Statistical analysis

Raw data from tail regeneration assay scoring was used to compare the extent of tail regeneration in control vs. treatments as previously described (Tseng et al., 2010). Comparison of two treatments was analyzed with Mann–Whitney *U* test for ordinal data with tied ranks, using normal approximation for large sample sizes. Multiple treatments were compared using a Kruskal–Wallis test with Dunn's Test corrected for tied ranks post-hoc. Data obtained from wound healing assays were analyzed using the Student's *t*-test.

3. Results

3.1. Tail regeneration

When tadpoles were exposed to MIT after tail amputation, they failed to regenerate tails (n > 30 per treatment, p < 0.01) (Fig. 2). Control, untreated tadpoles regenerated tails at almost 100% (Fig. 2A). Tadpoles treated with 50 μM MIT did not show an appreciable effect on either regeneration or development (Fig. 2B). However, 75 μM MIT and 100 μM MIT exposure both mostly blocked regeneration (Fig. 2C-D) (p < 0.01).

3.2. Effects on cellular regeneration

At 72 h post amputation (hpa), differences in nerve innervation, muscle formation, and notochord and spinal cord regeneration were examined using antibodies that identify these specific tissues (Fig. 3, dashed lines indicate amputation site, yellow arrowheads indicate missing tissues). Control untreated tadpoles with amputated tails showed spinal cord tissues extending into the regenerate (Fig. 3A1-2), whereas 75 μM MIT-treated tail stumps lacked spinal cord regenerative outgrowth (Figs. 3A3-4). Control tail regenerates also contained new muscle, whereas 75 μM MIT-treated tail stumps showed muscle degeneration at the amputation site (compare Fig. 3B1 and Fig. 3B3). Similarly, control tail regenerates contained new notochord tissues, but 75 μM MIT-treated tail stumps did not (compare Fig. 3C1 to Fig. 3C3). Lastly, control untreated regenerating tails showed strong nerve innervation into the newly growing tail tissues (Fig. 3D1). In contrast, 75 μM MIT-treated tadpoles showed a lack of similar innervation into the injury site (Fig. 3D3).

3.3. Temporal requirement for MIT

In the tail regeneration assay, there is a high level of mortality at the 100 μM MIT level (62% mortality compared to 7% for untreated controls, n ≥ 30 per condition) and most tadpoles did not survive for the duration of the seven-day assay. Tadpoles that had undergone tail amputation were placed together with tadpoles carrying intact tails into a 100 μM MIT environment. The uncut tadpoles showed low levels of lethality as compared to the injured tadpoles.

The kinetics of tail wound healing in both control untreated and MIT-exposed tadpoles were examined. At 3 hpa, control tadpoles showed no bleeding at the tail amputation site (Fig. 4A1). By 24 hpa, a regeneration bud was visible at the amputation site (Fig. 4A2) and a newly growing tail was clearly apparent by 72 hpa (Fig. 4A3).

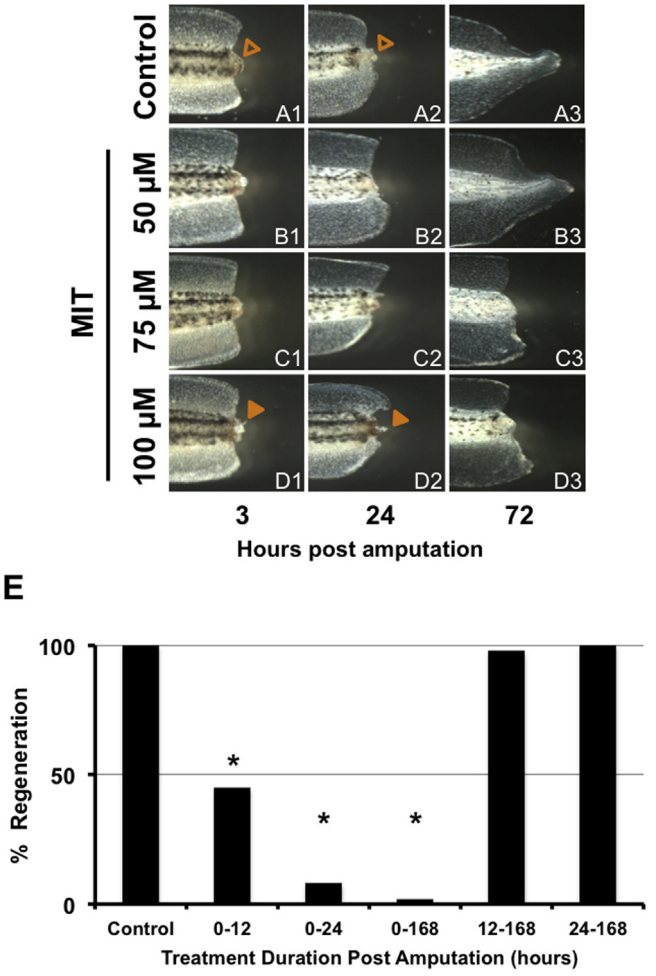

Fig. 4. Effects of MIT on Regeneration.
Tadpole tails were amputated at Stage 40 and treated for 24 h as follows: (**A**) untreated control, (**B**) 50 μM MIT, (**C**) 75 μM MIT, and (**D**) 100 μM MIT. All tails were imaged at the same magnification. (**E**) MIT was added to tadpoles after tail amputation for 12, 24, or 168 h; or were exposed to MIT starting at 12 or 24 hpa (n = 40 per treatment). Results were compared using a Kruskal-Wallis test. * denotes p < 0.01 as compared to untreated control.

None of the MIT concentrations tested altered blood clotting after tail amputation (data not shown). Furthermore, 50 μM MIT had no effect on wound healing following tail amputation (Fig. 4B1-3) and is similar to untreated controls (Fig. 4A1-3). In contrast, tadpoles with amputated tails treated with either 75 or 100 μM MIT continued to extrude cellular material from the amputation site even at 12 hpa (data not shown), indicating an impaired wound healing ability. By 24 hpa, tadpoles in 75 μM MIT had mostly completed their wound healing response – although with a significant delay and without the formation of a regeneration bud (Fig. 4C2). Tadpoles exposed to 100 μM MIT failed to close the wound site and cellular materials could be observed streaming into the clear medium at 24 hpa (arrows in Fig. 4D1-2). They also failed to form regeneration buds at the tail amputation site and no new tail tissues were regrown (Fig. 4D2-3). In addition, these tadpoles were unable to recover from the trauma, resulting in death.

After tail amputation, tadpoles treated with MIT from 0 to 24 hpa only showed strong inhibition of tail regeneration similar to those treated for the entire seven days (0–168 hpa) of the assay (n > 30 per treatment, p < 0.01). In contrast, treatment of MIT from 0 to 12 hpa was half as effective at inhibiting regeneration (Fig. 4E). Consistent with the early exposure results, addition of MIT at either

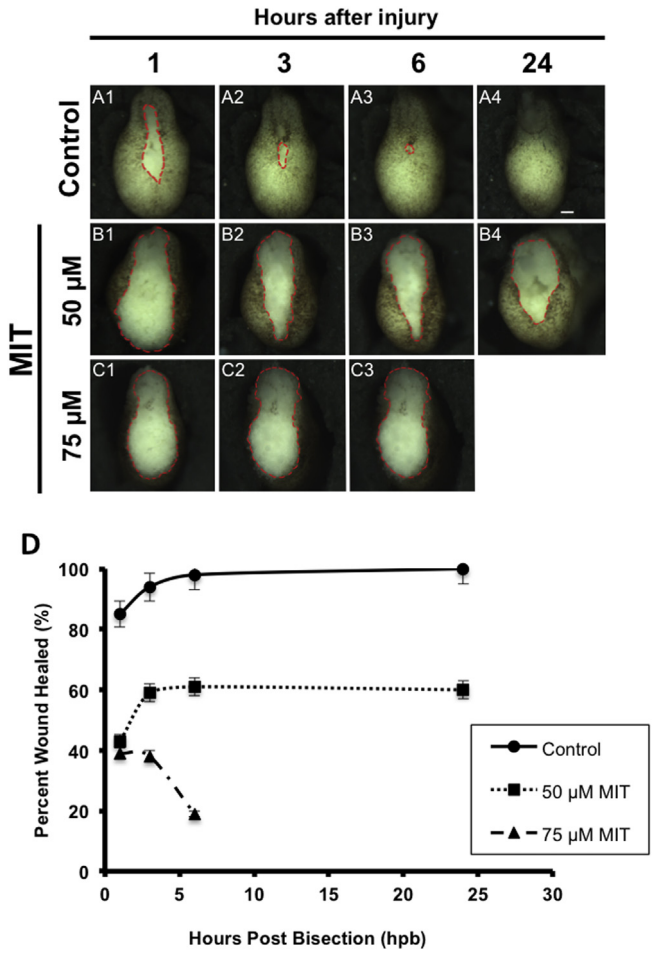

Fig. 5. Effects of MIT on Wound Healing.
Stage 25 embryos were treated as follows: (**A**) control, (**B**) 50 μM MIT, or (**C**) 75 μM MIT. Wound borders are outlined in red. Wounds at one hour of untreated embryos (**A1**), 50 μM MIT (**B1**), and 75 μM MIT (**C1**). Wounds at three hours of untreated embryos (**A2**), 50 μM MIT (**B2**), and 75 μM MIT (**C2**). Wounds at 24 hpa of untreated tadpoles (**A4**), 50 μM MIT (**B4**), and 75 μM MIT (**C4**); n = 10 per treatment, $p < 0.01$. Embryos shown in (**C3**) do not survive past six hours. (**D**) Quantification of wound closure area at same timepoints as shown in (**A-C**); n = 10 per treatment. Bissected embryos halves treated with 75 μM MIT failed to survive past six hours.

12 or 24 hpa failed to block regeneration, even when exposure was continued through day seven (Fig. 4E).

3.4. Wound healing in embryos

Stage 25 embryos were bisected (Fig. 5) and cultured in medium with or without MIT (n > 30 per treatment). At 1 h, the wounds of untreated embryos (Fig. 5A1) were significantly smaller than the initial wound area, whereas embryos treated with 50 μM MIT (Fig. 5B1) and 75 μM MIT (Fig. 5C1) retained the initial wound size. At 3 h, the wounds of untreated embryos (Fig. 5A2) have almost closed, while wounds of the embryos treated with 50 μM MIT (Fig. 5B2) show reduced wound area (7-fold larger than controls). However, the wound area of embryos treated with 75 μM MIT (Fig. 5C2) remain unchanged (11-fold larger than controls). By one day, untreated control embryo halves had completely closed the wound site (Fig. 5A4). Embryo halves exposed to 50 μM MIT showed partially closed wounds (Fig. 5B4), while those exposed to 75 μM MIT maintained large wound areas that remain largely unchanged (Fig. 5C1-3). 75 μM MIT halves, having failed to close their wounds, did not survive past six hours (Fig. 5D) ($p < 0.01$).

3.5. Antioxidants and MIT

Tadpoles treated with either GSH or NAC did not alter tail regeneration ability (Fig. 6). In the presence of 75 μM MIT, addition of either 75 μM GSH or 75 μM NAC successfully restored tail regeneration (Fig. 6A-B) (n > 30 per treatment, $p > 0.01$). The tail regenerates were also morphologically normal as compared to controls (compare Fig. 6C with E and F).

4. Discussion

In studies of aquatic toxicology, the main focus has been on understanding the effects of exposure on animal development. *Xenopus laevis* is highly suited for such studies because its development is well understood, facilitating quick identification of potential causes of defects from exposure. In this study, we show that *Xenopus laevis* is also an excellent model for studying the effects of chemical exposure on other key biological processes that are not part of normal developmental events. One of these is the injury response, a fundamental cellular process that is critical for organismal survival. We used the well-established *Xenopus laevis* tadpole tail, a complex structure that is vital for animal viability (Hoff and Wassersug, 2000). While there have been some studies on the effects of toxicants on fish fin regeneration (Verma, 2004; Zodrow, 2003), the effects on wound healing and tissue regeneration during development have not previously been examined in chemical exposure studies.

Previously, it was reported that MIT impaired neural development in tadpoles (Spawn and Aizenman, 2012). Here, our results showed that 75 μM MIT exposure effectively blocked tail regeneration by inhibiting the regrowth of nerve, muscle, notochord and spinal cord tissues in the tail (Figs. 2 and 3). In the tail regeneration assay, there is a high level of mortality at the 100 μM MIT level and most tadpoles did not survive for the duration of the assay. Two potential reasons may explain this high mortality rate. One possibility is that the 100 μM MIT exposure leads to general toxicity and subsequent death. A second possibility is that MIT treatment impairs wound healing and the subsequent continuous extrusion of cellular materials leads to death. Tadpoles that did not undergo surgery showed low levels of lethality as compared to the injured tadpoles at 100 μM MIT. This result indicated that general toxicity was not the main reason for tadpole mortality. A second possibility was that MIT exposure led to a wound healing defect.

At high MIT concentrations, tail wound healing does not occur as cellular material continues to be extruded from the wound site and resulted in tadpole lethality (Figs. 2 and 5). This observation is consistent with a previous study showing that tadpoles that fail to close the amputation site are unable to survive the injury (Ho and Whitman, 2008). At lower MIT concentrations, wound healing is delayed significantly and fails to complete within the normal 6–12 h time period (Fig. 4). This is consistent with other reports that show differential cellular responses to varying levels of MIT exposure (Ettorre et al., 2003). Moreover, failure to heal tail amputation wounds during the normal timeframe also prevented tail regeneration (Fig. 4). Together, our results revealed that MIT exposure prevents the normal wound healing after tail loss.

As *Xenopus laevis* is an aquatic model, we took advantage of the fact that chemicals can be introduced temporally into their media and removed by careful washes (Tseng et al., 2010). The temporal assay demonstrated that exposure to MIT for one day inhibits regeneration as well as treating tadpoles for the entire seven-day duration. Thus, a single one day exposure of MIT, early after injury, is sufficient to fully inhibit regeneration (Fig. 4E). MIT inhibited wound healing processes (and subsequent regeneration) only during the first day post injury, as later exposures have no effect (Fig. 4).

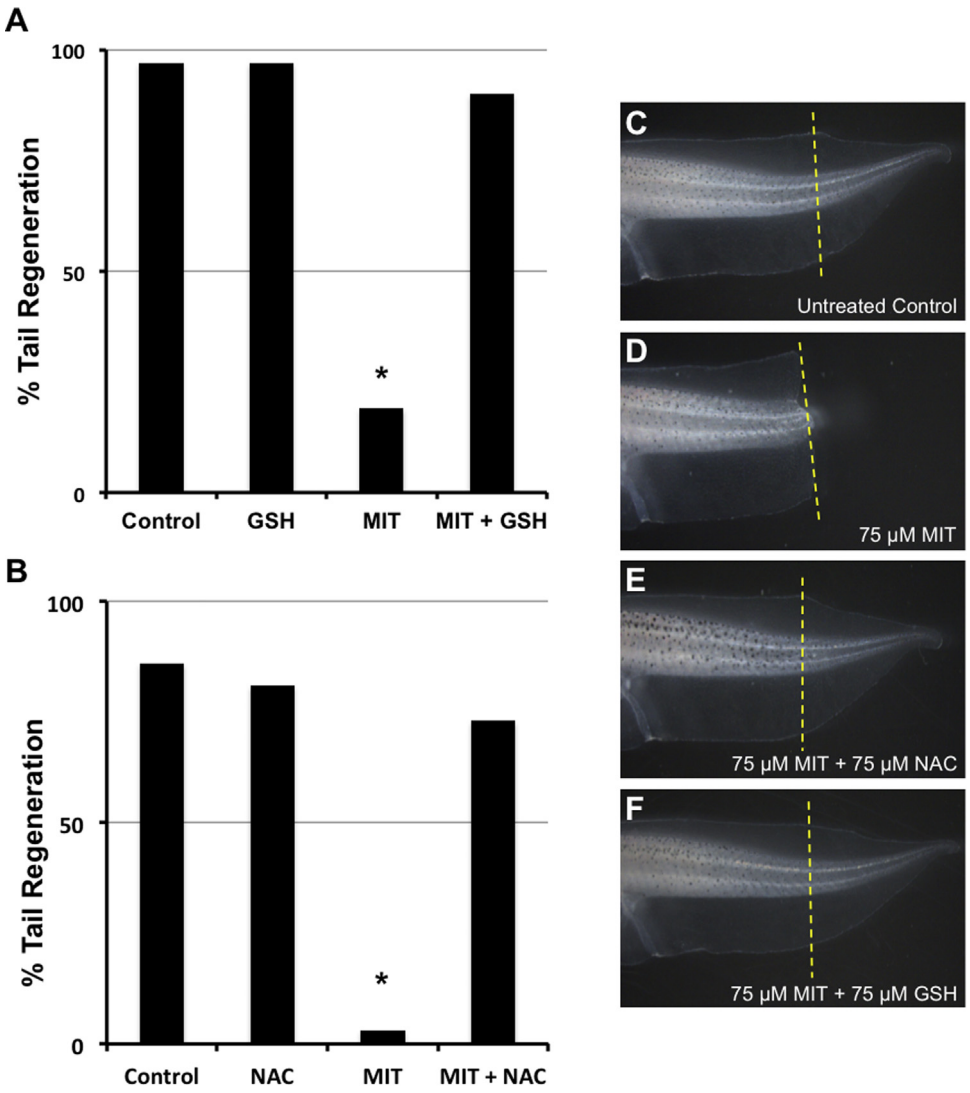

Fig. 6. Effects of Thiol-Containing Compounds on MIT Treatment.
(**A-B**) After tail amputation, tadpoles were either placed in normal media, or were treated with 75 µM MIT, 75 µM N-aceytl cysteine (NAC), 75 µM GSH, or a combination of MIT with either NAC or GSH; n = 30 per treatment. Tails were assessed for regeneration after seven days. (**D**) Tadpoles exposed to MIT. (**E-F**) Tadpoles treated with MIT in combination with NAC (**E**) or GSH (**F**). Dashed lines represent amputation planes. Results were compared using a Kruskal-Wallis test. * denotes $p < 0.01$ as compared to untreated control.

Together, these data suggest that the critical window for MIT exposure to affect regeneration is during the first day post injury.

Our results indicated that MIT may act to impair wound healing of the tadpole skin. Thus, the ability of embryos to heal an open wound in the presence of MIT was tested. Based on the results obtained (Fig. 5), we concluded that MIT acts to blocks wound healing. The data also showed that the consequences of wound healing failures can be severe. Failure to close wounds leads to lethality for the embryo and tadpole. Impairment of wound healing kinetics also blocked tail regeneration bud formation, which is critical for initiating the regeneration program. Thus, our results demonstrated that even if the tadpole can eventually heal its injury, a temporal delay is sufficient to block normal regenerative mechanisms from being activated. This suggested that wound healing must occur within a specific timeframe to enable regeneration and that regeneration is a temporal-dependent process.

The *Xenopus laevis* tail provides an excellent *in vivo* model system in which to elucidate the types of mechanisms that are disrupted by MIT action and to further understanding of its effects. MIT is a derivative of isothiazolinone, a common class of biocides that acts as a broad-spectrum antimicrobial agent and is used to control growth of bacteria, fungi, and algae in both consumer products and industrial settings (Lundov et al., 2011). MIT contains an active sulfur moiety that interacts with cysteine residues of available cellular protein thiols (Collier et al., 1990). It reacts with protein thiols *in vitro* (Frerot et al., 2013). Together with our data that MIT strongly inhibits wound healing *in vivo* (Figs. 1 and 4), these observations indicated that exposure to MIT may cause covalent modifications that alter protein function.

It has been shown that addition of thiol-containing reagents such as reduced glutathione (GSH) and N-acetyl cysteine (NAC) (both antioxidants) blocked MIT-induced tyrosine phosphorylation in monocytes (Bruchhausen et al., 2003). Once it reacts with a substrate, MIT is inactivated (Williams et al., 2014). Consistent with this mechanism, MIT exposure reduces intracellular GSH levels (Di Stefano et al., 2006; Du et al., 2002). Moreover, NAC can protect against reactive compounds (Rayburn and Friedman, 2010). We hypothesized that by using GSH or NAC to provide additional thiol groups to interact with MIT, these chemicals would protect thiol groups in critical cellular proteins, rescuing these substrates from

MIT modifications. Consistent with published reports, we showed that antioxidants containing thiol groups (such as GSH and NAC) protect tadpoles against the negative effects of MIT exposure and restore normal tail regeneration (Fig. 6). Interestingly, this protective effect occurs when MIT and the antioxidants are in a one to one molar ratio, supporting observations that MIT is indeed inactivated once it modifies a substrate molecule (Williams et al., 2014).

The restoration of MIT-inhibited tail regeneration by the addition of either GSH or NAC further suggests that thiol modifications can impair the wound healing process. Potentially, proteins involved in wound healing contain exposed thiol groups and thus are sensitive to MIT-induced modifications. For example, the activation of the transforming growth factor beta (TGF-beta) pathway is required for wound healing after tadpole tail amputation (Ho and Whitman, 2008). Free thiols can inactivate TGF-beta activity (Blakytny et al., 2006). Further studies to identify proteins targets that can be modified by MIT will help to delineate key mechanisms involved in inhibition of wound healing.

Additional mechanisms that have been shown to have protective effects against MIT are the zinc and extracellular signal-regulated kinase (ERK) pathways. TPEN, a zinc-chelating agent (Arslan et al., 1985), rescued MIT-induced neuronal cell death (Du et al., 2002). Similarly, two inhibitors of the ERK pathway, U0126 (Favata et al., 1998) and PD98059 (Alessi et al., 1995), also prevented MIT toxicity in neuronal cells (Du et al., 2002). We tested each of these inhibitors for their ability to rescue MIT-dependent block of tadpole tail regeneration and did not observe any phenotypic rescue (data not shown). This suggested that inhibition of zinc or ERK pathways may not be sufficient to protect against MIT effects during wound healing. It is likely that multiple mechanisms are disrupted during this process.

In this study, we showed that chemical exposure impairs aquatic animal development by inhibiting injury responses: wound healing and tissue regeneration. There may also exist additional exposure effects that are not readily observed in common assays but are nevertheless critical for animal health. It is likely that additional approaches (such as our injury response assays) are needed to assess the effects of chemicals on critical cellular responses required for non-homeostatic or developmental processes such as wound healing. As wound healing is a fundamental process, this *Xenopus laevis* model also provides additional advantages that will help to elucidate mechanisms of chemical toxicity and our understanding of tissue repair. Lastly, this study also has implications for considering the consequences of chemical runoffs and accumulation in the environment and their toxicity to other aquatic organisms.

5. Conclusions

Using the *Xenopus laevis* tail regeneration model, we identified defects in tissue regeneration and wound healing resulting from MIT exposure. This was an unexpected finding as there have been no reports to implicate MIT in the disruption of these processes. Our study confirmed a strong effect of MIT in blocking wound healing and tissue regeneration. Although these two processes are not part of the normal developmental events, wound healing and regeneration occur during development. The present study showed that MIT exposure can cause lethality in embryos and tadpoles by blocking the normal wound healing response. Additionally, MIT exposure at dosages that lead to delayed wound closure prevented tadpoles from regrowing tails. The disruption of wound healing and/or regeneration may affect proper development and impacts viability of the developing organism.

Author contributions

Conceived and designed the experiments: NDS SA JD YC AT. Performed the experiments: NDS SA YC JD JL. Analyzed the data: NDS SA YC JD AT. Contributed reagents/materials/analysis tools: JL AT. Contributed to the writing of the manuscript: NDS YC AT.

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