Critical Review Article

Title

Xanthine Oxidoreductase – A Novel Therapeutic Target for the Treatment of Chronic Wounds?

Melissa L Fernandez¹, Dario Stupar¹, Tristan Croll², David Leavesley¹,³,⁴ and Zee Upton¹,³,⁴

¹Institute of Medical Biology, Agency for Science, Technology and Research, Singapore
²Department of Haematology, Cambridge Institute for Medical Research, University of Cambridge, Cambridge, UK
³School of Biomedical Sciences, Faculty of Health, Queensland University of Technology, Brisbane, QLD, Australia
⁴Lee Koon Chian School of Medicine, Nanyang Technological University, Singapore.

Corresponding Author:

Dr Melissa Fernandez
8A Biomedical Grove.
#06-06 Immunos,
Biopolis 138648
Singapore
Phone - +65 6407 0051
Fax – +65 6464 2049
Email – melissa.fernandez@imb.a-star.edu.sg

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Abstract

Significance - Chronic wounds are a major burden to patients and to healthcare systems worldwide. These wounds are difficult to heal and treatment is often lengthy and expensive. This has led to research efforts focused on the wound environment attempting to understand the underlying pathological mechanisms of impaired wound healing. While, some of this research has translated to advancements in wound therapies and implementation of new treatment options, chronic wounds remain a significant challenge to treat. Thus, identification of effective, low-cost advanced wound therapies that enhance healing rates of these problematic wounds is still essential.

Recent Advances and Critical Issues – Xanthine oxidoreductase (XO), a molybdoflavin enzyme, is emerging as an important source of reactive oxygen species (ROS) in various pathologies, including diabetes and chronic wounds. XO has recently been shown to be upregulated in chronic wounds, stimulating the overproduction of ROS during dysfunctional wound healing. XO-induced ROS can amplify and potentiate inflammation in the wound environment further delaying wound closure.

Future Directions - The detrimental role of XOR in impaired healing indicates it may be a therapeutic target. Targeted inhibition of XOR has been shown to reduce the expression and activity of this enzyme in diabetic wound models. In turn, this resulted in a significant decrease in ROS levels in the wound environment and improved wound healing. Therefore, repurposing existing XOR inhibitors that are approved for human use may be able to restore homeostasis at the wound site and enable damaged tissue to return to normal healing.
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1.0 Scope and Significance

This review provides a brief overview of xanthine oxidoreductase (XOR), its structure and function. We then summarise the current evidence that suggests XOR may play an important role in impaired wound healing. In particular, we focus on the two products of XOR activity, ROS and uric acid, and how these factors may potentiate inflammation in non-healing wounds. Finally, we discuss the benefits of repurposing common XOR inhibitors that are approved for human use as a treatment for patients with hard-to-heal wounds.

2.0 Translation Relevance

Chronic wounds are characterised by an amplified and prolonged inflammatory phase. XOR is thought to play a key role in chronic wounds by generating excessive amounts of ROS and uric acid. Together these factors could stimulate inflammation and prevent proliferation, vascularisation and re-epithelialisation, thereby delaying the wound healing process. Inhibition of XOR therefore holds potential in preventing the overproduction of ROS and elevated levels of uric acid in the wound environment, restoring homeostasis and improving wound healing.

3.0 Clinical Relevance

The management and treatment of chronic wounds is one of the biggest health issues today, adding a significant financial burden to sufferers and to already critically stretched health care budgets. An unmet clinical need exists for effective, low cost advanced wound care therapies to improve healing. The inhibition of XOR may offer a novel cost-effective approach to treat a subset of non-healing wounds.
4.0 Background

4.1 Chronic wounds are a major burden worldwide

Chronic wounds are a significant and rapidly growing global health issue. The rising burden of diabetes, cardiovascular disease and obesity on an epidemic scale, in combination with a progressively ageing population, has led to the increasing incidence of these complex hard-to-heal wounds. The majority of chronic wounds present as leg ulcers with various aetiologies, but common manifestations include diabetic foot ulcers and pressure ulcers. It is estimated that approximately 1-2% of the population in developed countries will suffer from a chronic wound during their lifetime\(^1\). Common outcomes for these wounds are long term pain, loss of mobility, decreased quality of life, ongoing medical care and, frequently, limb amputation. Unfortunately, despite improvements in wound care and an increase in the variety of wound dressings and novel advanced wound therapies available chronic wounds remain a challenge to treat, highlighting that there is still a need for new therapies to improve healing rates.

4.2 Inflammation is a key feature of chronic wounds

Over the last two decades studies have demonstrated that the chronic wound environment is characterised by elevated levels of proteases such as matrix metalloproteinases (MMPs)\(^2\), reduced levels of protease inhibitors like the tissue inhibitors of metalloproteinases (TIMPs)\(^3\), and an abundance of inflammatory cells releasing excessive amounts of pro-inflammatory cytokines, the aforementioned proteolytic enzymes and toxic free radicals\(^4\). The combined effect of these factors results in accelerated degradation of the extracellular matrix (ECM)\(^5\) and growth factors\(^6\) and an amplified inflammatory state\(^4,7\). This leads to a decrease in cellular proliferation, inadequate vascularisation and the accumulation of necrotic tissue due to ischemia. This in turn encourages bacterial colonisation and can perpetuate the
inflammatory response, preventing wound repair\(^8\). While significant progress has been made to understand the processes involved in impaired wound healing, more research is required to elucidate the underlying causes of dysregulation in order to develop targeted therapies to treat these problematic wounds.

4.3 XOR - structure and function

The chronic wound environment is considered to be highly oxidising owing to the increased release of ROS. A large amount of ROS present in wounds are released by activated infiltrating neutrophils and macrophages undergoing “respiratory bursts” during phagocytosis\(^9\). Another potential source of ROS in the wound environment is the enzyme xanthine oxidoreductase (XOR)\(^{10}\). XOR is a complex molybdoflavin enzyme that is well-known for its role in catalysing the two terminal reactions in the purine metabolic pathway, namely the conversion of hypoxanthine to xanthine and subsequently xanthine to uric acid using either NAD\(^+\) or O\(_2\) (Figure 1)\(^{11}\). It is a homodimer with an approximate mass of 300 kDa, with each subunit consisting of a N-terminal 20 kDa domain containing two iron (Fe/S) centres, a central 40 kDa flavin adenine dinucleotide (FAD) domain and a C-terminal 85 kDa molybdopterin centre (molybdenum cofactor; Mo-Co) (Figure 2)\(^{12,13}\). The Mo-Co is the site of purine oxidation, while NAD\(^+\) and O\(_2\) are reduced at the FAD.

XOR exists in two inter-convertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). Under normal physiological conditions XOR is predominantly present in the dehydrogenase form and uses NAD\(^+\) as its preferred electron acceptor to yield NADH. Hypoxia, amongst other conditions, can lead to the conversion of XDH to the oxidase form, either by reversible sulfhydryl modification or by irreversible proteolytic cleavage\(^{12,14}\). Unlike the dehydrogenase form, XO is unable to bind NAD\(^+\) as these modifications obstruct
the binding site of the FAD region\(^{(12)}\). Instead XO consumes molecular oxygen to catalyse the conversion of hypoxanthine to xanthine and finally to uric acid, with concomitant production of superoxide (\(\text{O}_2^-\)) and hydrogen peroxide (\(\text{H}_2\text{O}_2\))\(^{(11, 15)}\) (Figure 1). Distinguishing between these two forms of XOR is challenging due to the lack of commercial antibodies that are specific for the dehydrogenase and oxidase forms. However, it is widely acknowledged that the oxidase form of the enzyme is the predominant source of ROS production and frequently associated with disrupting homeostasis in inflammatory disease states\(^{(11, 16)}\).

4.4 The potential role of XO in chronic wound healing

XOR has been shown to play a role in normal wound healing using an excisional murine wound model particularly during angiogenesis and keratinocyte proliferation\(^{(17)}\). These processes within the wound healing cascade are likely mediated by XO-derived ROS. At physiological levels ROS are known to shield the body against infectious agents and participate in numerous biological signalling pathways\(^{(18)}\). In contrast, XO expression and activity is significantly increased in tissue extracts from diabetic murine wounds when compared to wild-type/acute wounds\(^{(10)}\). This upregulation of XO activity was been shown to lead to an overproduction of free radicals in diabetic wound tissues, further prolonging wound closure\(^{(10)}\). \textit{In vitro} studies have shown that XO-derived ROS can also mediate the release of IL-1\(\beta\)\(^{(19)}\), a potent pro-inflammatory cytokine found to be elevated in wound fluid and tissue samples from chronic venous leg ulcers\(^{(20, 21)}\). Elevated levels of IL-1\(\beta\) can increase recruitment of immune cells to the site of injury further potentiating inflammation\(^{(22)}\). Finally, uric acid, the terminal metabolite of XO activity, has been shown to be elevated in wound fluids from chronic venous leg ulcers\(^{(23)}\). Elevated XO activity can lead to the sustained production of uric acid, which at high concentrations can crystallise into monosodium urate.
Fernandez (MSU). These urate crystals can trigger an inflammatory response stimulating the production of pro-inflammatory cytokines\(^{(19)}\), further perpetuating inflammation.

With this in mind, the following review examines the expression of XOR in normal and wounded skin and the role of XO in the chronic wound environment. In particular, we outline how excessive XO activity may play an important role in prolonging inflammation and delaying wound closure in a subset of chronic wounds. Finally, we discuss the relevance of specifically targeting XOR overactivity in the wound environment with common XOR inhibitors repurposed for the treatment of patients with hard-to-heal wounds.

5.0 Discussion

5.1 XOR expression is upregulated upon wounding

XOR is widely expressed in a range of organs, with the highest activity and expression found in the liver and intestines\(^{(22)}\). More recently, it has also been detected in normal intact murine skin, largely in the thin epidermal layer\(^{(17)}\). Upon wounding, XOR expression is upregulated at the wound edge in comparison to the surrounding intact tissue and the wound area. XOR immunoreactivity was also observed in cells in the peri-wound dermis and subcutaneous tissue; these were morphologically consistent with neutrophils. Immunofluorescence analysis of wounds fourteen days after wound closure showed that the neo-epidermal layer still expressed high levels of XOR and to a lesser extent the dermis also exhibited high levels of XOR\(^{(17)}\).

Other cell types in the wound environment, such as macrophages and endothelial cells have also been shown to express XOR \textit{in vitro}. XOR was detected in bone marrow-derived macrophages and primed human THP-1 macrophages +/- octacalcium phosphate (OCP)
crystal stimulation\(^{(19)}\). Furthermore, stimulation of bone marrow-derived macrophages with OCP crystals or alum increased the percentage of XO in these cells. Similarly, when exposed to hypoxic conditions, cultured endothelial cells exhibited increased XO expression, enhanced XO activity and the extracellular release of XO\(^{(24, 25)}\). XO released into the circulation can bind to negatively charged glycosaminoglycans (GAGs) on the luminal surface of vascular endothelial cells\(^{(26, 27)}\). The binding and sequestration of XO to GAGs increases local XO concentration and subsequent ROS production, thereby disrupting homeostasis by inducing oxidative stress and causing local tissue damage (Figure 3). Taken together, these observations indicate that XOR is highly expressed in cutaneous wounds throughout the healing process, with the majority of the expression confined to the keratinocytes and early inflammatory infiltrating cells\(^{(17)}\).

5.2 XO activity is elevated in chronic wounds in comparison to acute wounds

Chronic wounds result from disruptions of the normal wound healing process. Unlike acute wounds, chronic wounds remain stalled in the inflammatory phase, exhibiting elevated levels of inflammatory cells, pro-inflammatory cytokines and proteases\(^{(2, 7, 20, 21)}\). Pro-inflammatory cytokines in particular have been reported to stimulate the expression and activity of XOR in various tissues\(^{(28)}\). However, until recently there have been no studies that examined XO activity in acute or chronic wounds. Weinstein \textit{et al.} (2015) used a diabetic murine wound model to evaluate XO expression and activity in intact and wounded tissue. The study demonstrated that there were no differences in XO activity and expression in intact skin of both wild-type and diabetic mice. Wounding resulted in a three-fold increase in XO activity in wild-type mice compared to intact wild-type skin. In contrast, wounded diabetic mice exhibited a seven-fold increase in XO activity in the wound environment in comparison to intact diabetic skin. Interestingly, comparison of the two wound types demonstrated that XO
activity in the diabetic wound environment was approximately double that detected in the wild-type wound environment. Additionally a corresponding increase was observed at the mRNA level in diabetic wounds compared to wild-type wounds. These findings are consistent with research from our laboratory which found that XOR is upregulated in wound fluids from severe, non-healing venous leg ulcers in contrast to less severe, healing wounds\(^{(23)}\). Collectively, these results suggest that XO activity is increased following wounding but is significantly elevated in chronic wounds compared to acute wounds.

### 5.3 Elevated XO activity results in overproduction of ROS in the wound environment

Given that XO is a known source of free radicals, Weinstein et al. (2015) further examined if elevated XO activity may be responsible for the overproduction of ROS in the chronic wound environment. Using a DNA modification marker of oxidative stress and ROS levels, the authors confirmed that the increase in XO activity was accompanied by an overproduction of ROS in the diabetic wound site. The levels of ROS measured in diabetic wound tissue were significantly increased in comparison to intact diabetic skin and non-diabetic wound tissue. Moreover, selective inhibition of XO using siRNA led to a significant reduction in ROS levels, indicating that a significant proportion of ROS in the diabetic wound environment is generated by overactive XO at the wound site\(^{(10)}\).

The chronic wound environment is highly oxidising owing to the release of significant amounts of free radicals. Overproduction of free radicals can have deleterious effects in the wound environment, including lipid peroxidation and protein modification detected in wound fluids from chronic wounds\(^{(29, 30)}\). Post-translational modifications such as these alter the structure and function of proteins, increase their susceptibility to proteolysis, and frequently result in tissue damage\(^{(29)}\). A wound environment rich in oxidants can also lead to the
activation of redox-sensitive transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1)\(^{(31-34)}\). *In vitro* studies of redox regulation indicate that activation of these transcription factors up-regulates the expression of various genes involved in the inflammatory response, leading to the increased production of pro-inflammatory cytokines and MMPs\(^{(35, 36)}\). Indeed, stimulation by various pro-inflammatory cytokines, such as interferon (IFN), tumour necrosis factor-α (TNF-α) and interleukins (ILs), has also been shown to induce XOR expression and enzyme activity\(^{(28)}\). Thus, the evidence indicates that elevated XO stimulates the overproduction of ROS at the wound site, which in turn could disturb the redox homeostasis and further amplifying inflammation.

### 5.4 MUS crystals could further stimulate the inflammatory response in wounds

Uric acid is the other important by-product of the XO-catalysed purine metabolism pathway (Figure 1). The production of uric acid is associated with the release of free radicals, which in excess can cause cellular damage and further amplify inflammation as described earlier. Nonetheless, uric acid itself can trigger an immune response\(^{(37)}\). Using a transgenic uricase mouse model, Kono *et al.*, (2010) demonstrated that neutrophil infiltration and myeloperoxidase production was reduced in these transgenic mice upon cell death. However, this reduced inflammatory response was not replicated in uric acid-depleted mice stimulated with silica crystals, zymosan and LPS\(^{(37)}\). This suggests that these substances can trigger inflammation on their own, whereas the innate response to cell death is driven by uric acid. Therefore uric acid released upon cell death acts as an alarmin to trigger inflammation.

High concentrations of uric acid alone are incapable of inducing an inflammatory response *in vitro*\(^{(19)}\). This can only occur if monosodium urate crystals (MSU) are formed after large amounts of uric acid are released from dying cells. MSU crystals induce inflammation via the
activation of the NLRP3 inflammasome leading to the secretion of active IL-1β (Figure 4a)\(^{(38)}\). Indeed, bone marrow-derived macrophages exposed to MSU crystals secrete high levels of IL-1β, which is reduced upon exposure to uricase\(^{(19)}\). Secreted IL-1β as a result of inflammasome activation can potentially lead to increased recruitment of neutrophils and other immune cells to the site, again amplifying inflammation\(^{(39)}\) (Figure 4). Moreover, elevated levels of uric acid have been detected in wound fluid from venous leg ulcers with relative concentrations correlated with wound chronicity\(^{(23)}\). XOR is also present in elevated levels in wounds fluids obtained from these chronic wounds. Taken together these results suggest that elevated XO activity leads to overproduction of uric acid in the chronic wound environment. However, we have been unable to confirm the presence of urate crystals in the wound environment due to incompatibilities associated with sample collection and processing techniques. Similarly, in vitro studies have been unsuccessful in detecting urate crystal formation within dying cells or in cell culture supernatants\(^{(19)}\). Therefore, we hypothesise that the sustained production together with the accumulation of high levels of uric acid leads to the precipitation of urate crystals in the wound environment. These crystals can then activate the inflammasome leading to the secretion of IL-1β which serves as a potent signal of inflammation in the wound environment.

5.5 XOR is involved in crystal induced-IL-1β secretion

A common and well documented feature of chronic wounds is a sustained inflammatory response with continual infiltration of macrophages secreting elevated levels of pro-inflammatory cytokines\(^{(7,\ 20,\ 21)}\). IL-1β is a potent mediator of inflammation and is elevated in wound fluids and tissues from chronic venous leg ulcers\(^{(20,\ 21)}\). It elicits an inflammatory response by signalling through its receptor (IL-1R), attracting neutrophils and macrophages to the site of injury. Ligation of IL-1R has been shown to induce the production and secretion
of other pro-inflammatory cytokines, such as IL-6, thus perpetuating inflammation\(^{(40)}\). Therefore it is likely IL-1\(\beta\) is part of a positive feedback system that sustains inflammation in chronic wounds and contributes to impaired wound healing (Figure 4). Cultured murine bone marrow-derived macrophages elicit elevated IL-1\(\beta\) secretion upon stimulation with OCP crystals; this has been shown to be accompanied by a rapid increase in intracellular urate levels and XO activity\(^{(19)}\). This correlation was further validated with the use of XOR-specific inhibitors and targeted siRNA, both of which impair IL-1\(\beta\) secretion in macrophages before and after stimulation with monosodium urate (MSU), alum and OCP crystals. Furthermore, inhibition of XOR also reduced secreted caspase-1 and production of ROS in crystal-induced macrophages \textit{in vitro}\(^{(19)}\). These authors also demonstrated that the trigger for IL-1\(\beta\) secretion upon inflammasome activation is XO-derived ROS, suggesting that XO plays a role in caspase-1 processing, which in turn leads to IL-1\(\beta\) secretion in macrophages stimulated with crystals (Figure 4a).

5.6 Alternative emerging mechanisms of XOR

While studies suggest that elevated XO plays a role in dysfunctional wound healing, there is also evidence in the literature that demonstrates that XOR may play an important part in normal wound healing\(^{(17)}\). Using a murine excisional wound healing model, Madigan \textit{et al.}, (2015) demonstrated that inhibition of XOR with dietary tungsten significantly delayed wound closure and reduced wound angiogenesis and keratinocyte proliferation. Similarly, topically applied allopurinol, a common XOR inhibitor, reduced ROS production and significantly delayed wound closure. However, it is important to note that this study used a normal wound healing model to examine acute wound healing responses which are often severely dysregulated in chronic wounds. It is therefore unlikely that levels of XO expression and activity found in this acute model can be applied to chronic wounds. In fact, the authors
themselves state that XOR may be upregulated in impaired wound healing and targeting XOR under these circumstances maybe beneficial for patients with hard-to-heal wounds. This was substantiated by Weinstein et al., (2015) using a murine wound model to demonstrate that XO activity is pathologically elevated in diabetic wounds compared to wild-type wounds and that inhibition of XO improved wound closure\(^{(10)}\).

XO-mediated nitric oxide radical (\(\bullet\)NO) generation is another pathway that is attracting increasing attention\(^{(41)}\). There is evidence to suggest that XOR can function as a nitrite reductase, reducing nitrite (\(\text{NO}_2^-\)) to beneficial \(\bullet\)NO under anoxic conditions. This nitrite reductase activity of XOR could possibly be harnessed to increase \(\bullet\)NO production in wounds through \(\text{NO}_2^-\) supplementation, however, studies to date have been conflicting. *In vitro* supplementation of nitrate has been shown to significantly increase keratinocyte and endothelial cell proliferation\(^{(17)}\). These beneficial effects were significantly reduced when XOR was inhibited using allopurinol suggesting that nitrite acts via XOR to promote proliferation in these cells\(^{(17)}\). On the other hand, supplementation and depletion of dietary nitrate did not alter wound healing rates in murine excisional wound healing models\(^{(17)}\). Currently, there are no knockout models that can be used to provide insights into this XO pathway. Homozygous and heterozygous XDH knockouts in mice results in premature death making *in vivo* experimentation difficult and have hindered progress in this area\(^{(42, 43)}\).

### 5.7 Inhibition of XOR as a treatment for chronic wounds

As highlighted in this review, XOR-induced ROS production has the potential to amplify and potentiate inflammation in the wound environment, ultimately delaying wound closure. This suggests that XOR might be a good therapeutic target; the inhibition of this enzyme is therefore a potential novel approach to treat non-healing wounds. Targeted inhibition of XOR
has already been shown to have beneficial effects both *in vitro* and *in vivo*. As noted earlier, the treatment of diabetic wounds in mice with XDH siRNA was found to reduce wound damage caused by ROS and improved wound closure\(^{(10)}\). In addition, the inhibition of XOR in macrophages stimulated by crystalline activators *in vitro* also reduces inflammation by reducing the secretion of caspase-1 and IL-1\(\beta\)\(^{(19)}\). Thus, a therapeutic approach which attenuates XOR activity directly is also likely to decrease the amount of uric acid released *in situ*, and prevent the precipitation of pro-inflammatory MSU crystals in the wound site. Taken together these studies suggest that the targeted inhibition of XOR might help to restore homeostasis at the wound site and that processes which lead to tissue damage might return to normal healing.

XOR has been pharmacologically targeted to lower urate levels in patients with gout and hyperuricemia\(^{(11,16)}\). Various XOR inhibitors are therefore already approved for human use, making them suitable for reformulation to inhibit XOR in chronic wounds. Allopurinol is the XOR inhibitor most commonly used to treat gout and hyperuricemia. It is a non-competitive inhibitor and reacts with XOR to yield oxypurinol (alloxanthine), which in turn binds effectively irreversibly to XOR preventing further activity\(^{(11,16)}\). It is important to recognise that conversion of allopurinol to oxypurinol requires enzyme turnover, hence some ROS is produced before inhibition is accomplished. There are also some known adverse side effects of allopurinol when administered orally, including gastrointestinal intolerance, hypersensitivity reactions and skin rashes\(^{(11)}\). Nonetheless, allopurinol offers a number of advantages for reformulation as a wound therapy: it is cheap, off patent and can be readily monitored using its breakdown product oxypurinol. It has been formulated for a number of applications, including topically, intravenously and orally, as a gel, tablet, powdered-form and mouthwash\(^{(44-48)}\). Unlike systemic hyperuricemia, which is managed with oral therapy,
chronic wounds present a unique opportunity to directly target the disease site enabling a “site-specific” topical delivery approach. Topical application offers a number of other advantages, including ease of application, sustained high local drug concentrations, reduced risks of adverse drug interactions and direct access to the disease site. We propose that incorporating powdered allopurinol into a hydrogel will ensure a sustained drug release delivery mechanism. The active ingredient, in this case allopurinol, will gradually be released as the soluble fraction and adsorbed into the wound site.

XOR inhibitors have been used broadly to treat other XOR-related pathologies. Studies conducted in humans and animal models have reported positive results with the use of allopurinol in the treatment of corneal alkali burns\(^{49}\); in the prevention and treatment of chemotherapy tumour lysis syndrome\(^{48}\), radiation-induced mucositis and dermatitis\(^{45}\), and leishmaniasis\(^{46}\). In each of these clinical studies, the exact mechanism of action of allopurinol in these pathologies was not determined. However, clinical observations demonstrated that allopurinol treatment alleviated symptoms or facilitated return to tissue homeostasis. The treatment of chronic venous leg ulcers with a combination of allopurinol and compression therapy has also been shown to stimulate wound healing\(^{47}\). Crushed allopurinol tablets were administered daily for 7 days and thereafter once weekly until the end of the study at 12 weeks. Allopurinol significantly stimulated wound healing when compared to compression therapy alone with 50% of ulcers healed in 4 weeks and 93% of ulcers healed within 12 weeks\(^{47}\). The authors of this study suggest that oxygen-derived free radicals are implicated in venous ulceration and delayed wound closure, and that treatment with free radical scavengers like allopurinol stimulates wound healing. While the authors demonstrated that topical allopurinol improved wound healing, the amount of allopurinol used in the study is unclear, and the mechanism of action and supporting biochemical data
was not. Therefore, a more rigorous clinical trial is required to generate the critical data required to assess the clinical efficacy and long term safety profile of topical XOR inhibitors as new options for the management of chronic wounds. Moreover chronic wounds are clinically heterogeneous and multifaceted, hence, given the complexity of chronic wounds, it is unlikely chronic inflammation associated with elevated XOR activity is the underlying cause of all hard-to-heal wounds.

Hyperuricemia, or excessive levels of uric acid, can develop as a consequence of elevated XOR activity. Therefore, measures of uric acid in wound fluid and tissues may hold potential as a biomarker to identify hard-to-heal wounds with elevated XOR activity. Such a diagnostic tool may, for example, enable clinicians to select patients that would be benefit from therapies directed at dampening wound inflammation, such as those involving inhibition of XOR. Uric acid could also be used as a prognostic marker to monitor the effectiveness of XOR inhibitor treatments and response in individual patients. Developing a diagnostic test for this purpose should be straightforward. Uric acid is already measured in biological samples, including plasma and urine, using commercially-available assays. Sophisticated sensor-based technologies are also in development and these maybe suitable for incorporation into various dressing materials to measure uric acid at the wound site\(^{(50)}\). The development of simple and convenient diagnostic tests could therefore be a major step to assist in the prognosis and management of chronic wounds and potentially also guide therapy.

6.0 Summary

In summary, XO activity is significantly elevated in chronic wounds which results in an overproduction of ROS disrupting homeostasis and impairing wound healing. In common with increased ROS, levels of uric acid can also increase as a consequence of elevated XOR
activity. The release of large amounts to uric acid in the wound environment can potentially crystallise to MSU, triggering inflammasome activation and IL-1β secretion. Furthermore, XO has been shown to mediate IL-1β secretion upon inflammasome activation in cells stimulated with crystals. Elevated levels of pro-inflammatory cytokines, in particular IL-1β, can further amplify inflammation through the recruitment of inflammatory cells. These immune cells can secrete pro-inflammatory cytokines, proteases and release ROS into the wound environment amplifying the inflammatory state. Despite these deleterious effects, XOR-mediated nitrite conversion to nitric oxide is emerging as a beneficial pathway. However, studies to date are conflicting and more research is required to elucidate the regulation of XOR and its interaction with other cellular pathways during normal and impaired wound healing.

Targeting the XOR enzymatic pathway to reduce uric acid and ROS generation in situ would appear to be beneficial for chronic wounds. A range of existing XOR inhibitors could be reformulated and trialled as novel wound therapies. However, currently no suitable animal models of chronic wounds that also include neuropathy or vascular insufficiency exist, necessitating direct human clinical trials to determine the safety and efficacy of these treatments. While challenging, the outcome of these studies could deliver diagnostic and companion assays and novel therapeutics enabling individualised treatment for patients with chronic wounds.

7.0 Take-Home Messages

- XOR expression and activity is upregulated in the chronic wound environment.
- XOR may be responsible for the overproduction of ROS resulting in oxidative stress at the wound site and prolong wound closure.
• XOR may play an important role in sustaining inflammation in chronic wounds by regulating IL-1β secretion upon inflammasome activation.
• Sustained XOR-mediated uric acid production could precipitate to form urate crystals that can further stimulate the inflammatory system.
• XOR inhibition could potentially reduce excess uric acid and ROS generation, thus restoring the wound bed environment to a healing phenotype.

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10.0 About the Authors
Melissa Fernandez, PhD, is a Senior Research Fellow within the Tissue Technologies (TT) research group at the Institute of Medical Biology, Agency for Science, Technology and Research (A*STAR). Her research focuses on identifying the underlying causes of inflammation in chronic wounds, in particular venous leg ulcers. Dr Dario Stupar is a Research Fellow within the Tissue Technology research group at the Institute of Medical Biology, A*STAR. His research focuses on identifying factors in wound fluid that sustain inflammation. Dr Tristan Croll is a Research Fellow in structural biology in the Read Laboratory at Cambridge Institute for Medical Research, Cambridge UK. A/Prof David Leavesley is a Senior Principal Investigator and co-leader of the Tissue Technology research
Fernandez

group at the Institute of Medical Biology, A*STAR. His interests are in the interactions of cells with their microenvironment. Professor Zee Upton is a biochemist. She is Research Director and co-leader of the Tissue Technology research group at the Institute of Medical Biology, A*STAR, a research team focused on adopting interdisciplinary approaches to improve wound and tissue repair.

11.0 Abbreviations and Acronyms

H$_2$O$_2$ – Hydrogen peroxide

O$_2$ - Oxygen

O$_2$• - Superoxide

NO$_2$• - Nitrite

’NO – Nitric oxide

AP-1 – Activator protein - 1

ATP – Adenosine triphosphate

DNA - Deoxyribonucleic acid

ECM – Extracellular matrix

FAD – Flavin adenine dinucleotide

GAG - Glycosaminoglycans

IFN – Interferon

IL - Interleukin

LPS - Lipopolysaccharide

MMP – Matrix metalloproteinases

Mo-Co – Molybdenum cofactor

MSU – Monosodium urate

NAD - Nicotinamide adenine dinucleotide
NADPH - Nicotinamide adenine dinucleotide phosphate

NF-κB - Nuclear factor-κB

OCP – Octacalcium phosphate

RNA - Ribonucleic acid

ROS – Reactive oxygen species

TIMP – Tissue inhibitors of matrix metalloproteinases

TNF – Tumour necrosis factor

XDH – Xanthine dehydrogenase

XO – Xanthine Oxidase

XOR – Xanthine Oxidoreductase
12.0 References


Figure Legends

**Figure 1 – The Purine Metabolic Pathway** – Adenosine triphosphate (ATP) is sequentially degraded to hypoxanthine and xanthine which are substrates of XOR. Guanine can also be converted to xanthine increasing substrate availability for XDH/XO. XOR exists in two interconvertible forms, the dehydrogenase form (XDH) which reduces NAD$^+$, while the oxidase form (XO) of the enzyme reduces oxygen, concomitantly producing superoxide and hydrogen peroxide. The oxidase form of the enzyme is the predominant source of ROS production and is associated with disrupting homeostasis in inflammatory pathologies, including chronic wounds. Both forms of XOR catalyse the conversion of hypoxanthine to xanthine and finally xanthine to uric acid. In humans, uric acid is the terminal metabolite in the purine metabolic pathway and is found in elevated levels in wound fluid from chronic wounds. (Reproduced from Pacher *et al.*, 2006)

**Figure 2 – The Structure of Bovine Xanthine Oxidoreductase** - The 3D model illustrates bovine xanthine dehydrogenase (XDH), the best available structure and analogous to the human form of XOR. It exists as a homodimer of approximately 150 kDa subunits with the three major domains and two connecting loops. The domains are a N-terminal 20 kDa domain containing two iron (Fe/S) centres (red), a central 40 kDa flavin adenine dinucleotide (FAD) domain (green) and a C-terminal 85 kDa molybdopterin centre (molybdenum cofactor; Mo-Co) (blue). The loop connecting the Fe/S domain with the FAD domain is shown in yellow and the one connecting the FAD domain with the Mo-Co is depicted in brown. The Mo-Co is the site of xanthine and hypoxanthine oxidation, during which electrons that are transferred to molybdenum are then transferred to FAD via the two Fe/S centres. The electron acceptors, NAD$^+$ and O$_2$, are subsequently reduced at the FAD site. (Reproduced from Enroth *et al.*, 2000)
Figure 3 – The Proposed Mechanism of Action of Circulating XOR – Hypoxia and pro-inflammatory cytokines induce xanthine dehydrogenase (XDH) expression in tissues and vascular endothelial cells. XDH is released into the circulation where it is rapidly converted to xanthine oxidase (XO) and sequestered by negatively charged glycosaminoglycans (GAGs) on the surface of vascular endothelial cells. The binding and sequestration of XO by GAGs concentrates local XO-mediated reactive oxygen species (ROS) production, which can disrupt homeostasis. (Reproduced from Cantu-Medellin and Kelley 2013)

Figure 4 – Proposed XOR-Mediated Inflammasome Activation Pathways – (a) XOR-catalysed production of uric acid can lead to hyperuricemia and precipitation of urate crystals. These crystals are phagocytosed, resulting in the activation of the NLRP3 inflammasome. The activation of NLRP3 by either pathway results in the activation of caspase-1, which cleaves proIL-1β to its active form. Secreted IL-1β can act as an attractant for neutrophils and other immune cells and can lead to the upregulation of other pro-inflammatory cytokines. This positive feedback mechanism of IL-1β can sustain inflammation in chronic wounds and impair wound healing. (b) XOR-mediated production of ROS triggers IL-1β secreted upon stimulation with crystal DAMPS. (Reproduced from Shi 2010)