Pathological Society Report

# Why do patients on nicorandil therapy develop non-healing ulcers?

## Introduction

There are four overlapping phases of wound healing: haemostasis, inflammation, proliferation and remodelling (Maxson et al., 2012). Haemostasis involves the aggregation of platelets to form a primary clot and activate the coagulation cascade (Diegelmann & Evans, 2004). The inflammatory phase brings along leukocytes, primarily neutrophils followed by macrophages, which are required to remove cellular debris as well as any infectious agents (Eming et al., 2007). The proliferative phase involves the formation of granulation tissue and consists of three processes: fibroblast proliferation, ECM synthesis and angiogenesis (Bauer et al., 2005). Transforming growth factor beta (TGF-β) secreted from macrophages stimulate fibroblast differentiate into myofibroblasts which provide contracture, pulling in the edges of the wound (Pang et al., 2017). The final phase of wound healing involves remodelling the makeup of the ECM and the replacement of collagen III with collagen I.

One of the first symptoms of ischaemic heart disease is angina pectoris. A disproportion in supply and demand of oxygen results in transient episodes of ischaemia to the heart (Hiremath et al., 2010). Nicorandil is a popular drug of choice amongst cardiologists. It has been available in the UK since 1994; 3 years after its release, reports of oral ulceration were associated to its use (Reichert et al., 1997), followed by other gastrointestinal (GI) lesions (Pisano et al., 2016). There is a considerable variation in onset time for ulcers: they can take a few weeks to several years to develop. However, those affected are often on a dose of at least 20 mg twice a day, or have recently had an increase in dose (Patel & Harding, 2010). These non-healing ulcers will only resolve with a reduced dose or complete withdrawal of nicorandil (Watson et al., 2004).

Nicorandil is a potassium channel opener which works through two mechanisms giving it the ability to dilate both arteries and veins (Hiremath et al., 2010). The activation of potassium channels leads to calcium channel blockade and the dilation of arteries; while a nitrate moiety stimulates the increase in cyclic guanosine monophosphate (cGMP), resulting in the vasodilation of venous vessels (Scully et al., 2001). Hepatic metabolism accounts for the breakdown of nicorandil. Interestingly though, there is no evidence to suggest for worsened adverse drug reactions (ADR) in patients with liver failure (Pisano et al., 2016). There are two main metabolites: nicotinamide and nicotinic acid – these merge into the endogenous pool of nicotinamide adenine dinucleotide / phosphate (NAD/NADP).

The mechanisms by which nicorandil causes these non-healing ulcers is poorly understood, but there have been several hypotheses put forward. However, few come without contradicting evidence.

A common theory involves a vascular steal phenomenon (Watson et al., 2004). Vasodilation of the coronary arteries causes a reduced blood supply elsewhere in the body, resulting in a delayed healing process. However, the oral mucosa is well vascularised and not a watershed area (Scully et al., 2001), rendering this theory unlikely.

Excessive activation of K+ channels can result in disproportionate levels of electrolytes (Chowdhry & Mohanty, 2015), potentially causing a local toxic action. However, this does not explain the delayed onset of ulcers as the potassium channels are affected shortly after the absorption of nicorandil.

A direct local toxic effect caused by nicorandil or its metabolites is a comply put forward hypothesis as to the aetiology of the ulcers. Repeated ingestion of nicorandil results in the NAD/NADP pool becoming saturated, resulting in a gradual accumulation of nicotinamide and nicotinic acid. It appears these two metabolites work together to cause ulceration (Trechot et al., 2015). However, nicotinic acid is also associated with hepatotoxicity, yet there are few cases of this as an ADR (Pisano et al., 2016).

It has been theorised that myosin is dephosphorylated in a dose-dependent manner (Patel & Harding, 2010); hindering the contraction of actin filaments which is necessary for fibroblast contraction as well as cell migration into the wound to repair mucosal microtrauma. Although, if this were the case, then surely nicorandil would only delay, not halt, the healing process.

The majority of these hypotheses involve fibroblasts in one way or another. However, there has been very little to no research on how nicorandil may affect the activation of fibroblasts into myofibroblasts.

## Aims and objectives

This project hypothesised that nicorandil reduces the activation of fibroblasts which impedes the healing process. The project aims were:

To assess the effect of treating oral fibroblasts with nicorandil by activating the cells and then, in the presence and absence of nicorandil measure:

1. Changes in protein expression associated with fibroblast activation induced by TGF-β1, angiotensin II and endothelin I
   1. Alpha smooth muscle actin (α-SMA)
   2. Fibronectin extra domain A (FN-EDA)
   3. Collagen 1 (col 1)
2. Fibroblast contractility

## Methods

Primary normal oral fibroblasts (NOF) were isolated from wisdom tooth extractions at the Charles Clifford Dental Hospital. They were cultured in fibroblast growth medium. Fibroblasts were treated with TGF-β1 (5 ng/ml) for 48 hours and their activation profiles were assessed. A mortality assay (MTS assay) was used to create a survival curve for nicorandil at several doses: (0, 0.1, 0.3, 1, 3, 10, 30 µM) for 48 hours. Four of these doses (0, 1, 10 and 30 µM) were used to treat inactivated NOFs. NOFs were then treated with 10 µM nicorandil in conjunction with endothelin 1 (ET-1), angiotensin (Ang II) and TGF-β1.

Activation profiles were investigated through several mechanisms. Quantitative polymerase chain reaction (qPCR) was used to look at gene expression for three genes: α-SMA, fibronectin (FN-EDA), and collagen 1 (col 1). Western blots and immunofluorescence looked at protein expression of α-SMA. Finally, fibroblasts were cultured in a 3D collagen matrix and contractility assays were carried out as a functional assay. All experiments had three biological repeats unless otherwise stated.

## Results

Firstly, myofibroblasts were characterised with TGF-β1 treatment. qPCR showed a significant increase in α-SMA, col 1 and FN-EDA gene expression; all markers of fibroblast activation. Western blots also showed a profound increase in α-SMA protein expression. TGF-β1 is therefore successful in activating fibroblasts into myofibroblasts.

An MTS assay showed no significant decrease in cell survival; all samples were over 90% viable. Therefore, any changes in gene or protein expression are down to a change in activation profiles, rather than a decrease in cell number. Treatment with four doses of nicorandil showed no significant changes in gene expression of α-SMA, FN-EDA or col 1 through qPCR. Western blots showed a potential decrease in α-SMA protein at the highest of doses (30 µM) but the remaining doses appeared unchanged. At a dose of 10 µM of lower, nicorandil does not have an effect on the expression of these activation markers; in future experiments, nicorandil was used at this dose.

Fibroblasts were activated with several growth factor treatments (TGF-β1, ET-1 and Ang II) in the presence and absence of nicorandil. There were no significant changes in the gene expression of α-SMA, col 1 or FN-EDA when nicorandil was included. However, the expression of α-SMA appears to have decreased considerably with TGF-β1 when nicorandil was included. Further biological repeats may narrow the error bars, possibly reaching a significant p-value. A significant decrease in fibroblast function was seen in a contractility assay with a p value of 0.0286, although this was only one biological repeat due to time constraints. Further repeats would be required to confirm this significance. This assay was arguably the most important in the study as it is a functional assay. The 3D collagen culture also better represents the in vivo environment of fibroblasts and is therefore more valuable over 2D culture on plastic.

## Conclusions

This study has confirmed that activation of myofibroblast phenotype from fibroblasts results from treatment with TGF-β1, ET-1 and Ang II as seen by the increased expression of α-SMA, col 1 and FN-EDA. Nicorandil does not appear to affect gene expression of these same activation markers; however, it does decrease the contraction of myofibroblasts – a response essential for wound healing. Further investigations are needed into altered fibroblast activation profiles in the presence of nicorandil when cultured in 3D.