Effect of diclofenac on TNF α and HIF1 α levels in rat supraspinatus tendon repair

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Summary

Introduction: Tendon healing is influenced by proinflammatory cytokines such as tumour necrosis factor alpha (TNF α) and hypoxia-induced factor 1 alpha (HIF1 α). We hypothesise that diclofenac effects on HIF1 α and TNF α levels during healing process.

Methods: A supraspinatus tear model was created and repaired to its anatomic footprint with 42 male Wistar rats. Rats were randomized to study and control group. Diclofenac 1 mg/kg/day was administered to the study group in a subcutaneous way. The rats were killed at weeks 1, 3 and 6, and seven rats from each group underwent immunohistochemical examination with TNF α and HIF1 α .

Results: HIF1 α was highest in the first week in both diclofenac and control group. HIF1 α levels decreased significantly at the 6th week in the control group ($p=0.011$) but in diclofenac group this decrease was observed at the 3rd week ($p=0.024$). TNF α levels decrease significantly at 3rd week in both groups ($p<0.001$), but in diclofenac group TNF α level was high at 6th week compared to 3rd week ($p=0.051$).

Conclusion: Diclofenac could have effect on HIF1 α and TNF α which are important in different phases of tendon healing.

Level of evidence: basic science study.

KEY WORDS: tumour necrosis factor-alpha, rotator cuff, diclofenac, hypoxia-inducible factor 1 alpha, inflammation.

Introduction

Rotator cuff tears are a major cause of shoulder pain and dysfunction. Despite surgical treatment, recurrence rates are very high in massive tears¹. Early retear rates could be up to 40% in large tears in one year². Although the size of tear is associated with retear rate, it is mainly a multifactorial process³. Hypoxia, ischaemic damage, oxidative stress, hyperthermia, impaired apoptosis, inflammatory mediators, fluoroquinolones, and matrix metalloproteinase imbalance have all been implicated as mechanism of retear and tendinopathies⁴.

NSAIDs are widely prescribed and are used to treat muscle injuries over the years. Among non-steroidal drugs, diclofenac sodium is a non-narcotic pain reliever with the most potent analgesic effect⁵. Diclofenac inhibits the inflammatory response during the tendon healing process via blocking phosphoglandin synthesis from arachidonic acid⁶. Besides NSAIDs could also affect the biomechanical outcomes of tendon healing⁷.

Tendon healing is probably influenced by the presence of pro-inflammatory cytokines such as TNF α . TNF α is one of the most potent proinflammatory cytokine especially in mechanical load and the injurious stimuli⁸. TNF α is a cytokine with diverse functions that is produced by transmembrane protein and cleaved to release soluble protein. TNF α signalling occurs via two structurally related but functionally distinct receptors; TNFR1 induces apoptosis and proinflammatory effects whereas TNFR2 is related to tissue repair, growth-modulating effects, and differentiation⁹.

HIF1 α is recognised to control key aspects of inflammation, particularly leucocyte recruitment and subsequent cytokine production¹⁰. HIF1 α regulates tendon regeneration and cell differentiation to tenocytes in tendon healing¹¹. HIF1 α also induces the expression of several genes which have beneficial effects on the regeneration and protection of many organ systems, including neural tissue, heart, lung, and skeletal muscle¹². HIF1 α is an important transcription factor for angiogenesis. Hypoxia initiates intracellular stabilisation of HIF1 α , and HIF complex binds to the hypoxic response element in the promoter region of VEGF gene and induces transcription of genes¹³. Inhibition or ablation of HIF-1 signalling has been shown to reduce angiogenesis and collagen deposition in some fibrotic models¹⁴.

Our hypothesis was HIF1 α and TNF α levels will decrease as the inflammatory phase will end, and as diclofenac blocks the inflammatory response, this de-

crease in the HIF1 α and TNF α levels should be more rapidly.

Materials and methods

Approval of the Local Ethical Board for Animal Experiments was obtained prior to the study. Principles of laboratory animal care were followed. The study meets the ethical standards of the Journal¹⁵. A rat model was preferred since the rats have a supraspinatus tendon which passes under an arch similar to that of the human supraspinatus tendon. The study included 42 male Wistar rats weighting 200-300 g. All rats underwent a surgical procedure.

Rats were randomized to the study group (n=21) or the control group (n=21). Then, the groups were divided into three subgroups, representing periods of 1, 3 and 6 weeks. Daily subcutaneous diclofenac sodium 1 mg/kg was administered to the rats as same as the human therapeutic dose in the study group. The rats in the control group received no additional treatment after the surgery. At the end of the respective follow-up period, all rats were killed, and 7 rats from each subgroup were used for histopathological analysis.

Surgical procedure

Prophylaxis was performed using a subcutaneous injection of gentamicin 8 mg/kg before the surgery. Surgical procedures were carried out under intraperitoneal anaesthesia with a mixture of 50 mg/kg Ketamine-HCl (Alfamine, Alfasan, TR) and 10 mg/kg Xylazine HCl (Alfazyne, Alfasan, TR). The surgical area was cleaned prior to the surgery. All surgical procedures were performed under aseptic conditions. A 1 cm transverse skin incision was made from the lateral border of the acromion. A portion of the spinodeltoid muscle was detached from the acromion. The acromion was gently retracted to expose the

supraspinatus tendon, and the tendon was removed from the attachment point to the tuberculum majus underneath the acromion using the tip of a No. 15 scalpel after placement of a sling suture. The footprint of tendon debrided. Then, the supraspinatus tendon was repaired by suturing it to the original position transosseously with a 4/0 polyester suture. There was no restriction of movement during the post-operative period.

The rats were killed at post-operative weeks 1, 3, and 6. The humerus and supraspinatus muscle dissected from the rest of the body to form a tendon-humeral unity after exposing the acromial arc without giving any harm to the healing portion of the tendon¹⁶.

Histology

After the specimens prepared as a tendon bone unit were fixed in 10% formaldehyde solution, they were decalcified in 20% formic acid for 1 day. All specimens were examined in coronal section plane in two 3 mm-thick slices, and embedded in paraffin. Five-micron-thick sections were cut, and stained with hematoxylin and eosin (HE). For examining vascular proliferation, a 30.000 micron square meter was chosen at the tendon bone healing region where maximum vascularity exists. Newly formed vessels were counted and 0-5 vessels classified as 1, 6-20 classified as 2 and above 21 as 3^{16,17}.

Immunohistochemical analysis

For IHC examinations of muscle-tendinosis tissue sections, TNF α [4E1]:sc-130349 Santa Cruz biotechnology, Inc Santa Cruz, California, USA and hypoxia-induced factor 1 α [(HIF 1 α : H α):sc53546. Santa Cruz biotechnology, Inc Santa Cruz, California, USA] density and intensity of dye uptake of the TNF α antibody was evaluated as described by Khandoga et al.¹⁸. TNF α was evaluated as no staining (-), slight staining (+) and intense staining (++) (Fig. 1). IHC findings according to the groups were

Figure 1. 2+ cytoplasmic staining for TNF α at healing area at 3 weeks. White arrow heads show the cytoplasmic staining of TNF α .

Figure 2. 4+ cytoplasmic and nuclear staining for HIF1 α at healing area at 3 weeks. Blue arrow heads show the nuclear staining of HIF1 α .

calculated as 0 points for no staining (-), 1 point for slight staining (+), and 2 points for intense staining (++). HIF-1 α was evaluated as (+) for 1-25% staining, (++) for 26-50% staining, (+++) for 51-75% staining and (+++++) for 76-100% staining and they were given degrees as 0, 1, 2, 3 and 4 respectively (Fig. 2). Cytoplasmic staining for TNF α and nuclear and cytoplasmic staining for HIF1 α was accepted as positive. All pathologic examinations were performed by pathologists blinded to the groups using light microscopes.

Statistical analysis

SSPS version 20 software package was used for analysis of data. For histological and immunohistological assessments one way ANOVA test with post-hoc

Bonferroni multiple comparisons was used. For correlations of vascular proliferation with HIF1 α spearman correlation test was used. A p -value <0.05 was considered significant.

Results

There was no loss in rats during the postoperative period. No infection was observed in injection sites and surgical site.

HIF1 α was highest in the first week in both diclofenac and control group. HIF1 α levels decrease significantly at 6th week in control group ($p=0.011$) but in the diclofenac group, this decrease was observed at 3rd week ($p=0.024$) (Fig. 3).

Figure 3. Changes in the mean HIF1 α levels by the time in both groups.

* represents the significant decrease in the HIF1 α at the 3rd week and † the significant decrease in HIF1 α levels at the 6th week.

There was a moderate correlation between HIF1 α and vascular proliferation in control group ($p=0.008$ Rho=0.559) but this correlation was not observed in diclofenac group ($p=0.255$, Rho=0.260).

The vascular proliferation was highest at the 1st week in the control and diclofenac group but was significantly low in the control group than diclofenac. Then vascular proliferation decreased in both groups and there was no significant difference between groups at 3rd and 6th weeks ($p=0.03$, $p=0.682$, $p=0.73$ respectively).

TNF α levels also were highest at the first week in both diclofenac and control group and decrease significantly at the 3rd week in both groups ($p<0.001$), but in the diclofenac group, TNF α level was high at 6th week compared to 3rd week ($p=0.05$) (Fig. 4).

Discussion

Our key finding is that there is a difference in cytokine levels between the diclofenac group and control and the levels were decreased with time. One of the important finding of our study is HIF1 α and TNF α levels were highest at the first week and significantly decreased by the weeks. In a rat tendon experiment model, HIF1 α levels were analysed at 0, 7, 14 and 28 days. HIF1 α levels significantly increased immediately after surgery (1.53 fold) when compared to control group, then a significant decrease was observed at 14 and 28 days¹⁹.

HIF1 α is normally hydroxylated in presence of oxygen, iron and 2-oxoglutarate. After a trauma, a hypoxic environment occurs at the injured area. Hypoxia initiates intracellular stabilization of HIF1 α . HIF1 α reacts with von Hippel Lindau protein and then undergoes ubiquitination and destroyed. In hypoxia, the oxygen required for HIF1 α to be ubiquitinated is missing. Thus HIF1 α persists intact, moves to nucleus

and recruits coactivator proteins to HIF binding site. The results are up-regulation of a large number of target genes and VEGF²⁰. These genes play an important role in wound healing and tissue repair via pro-inflammatory, angiogenic and potentially profibrotic process²¹. So the highest of HIF1 α levels with a correlation of vascular proliferation at the first week describes the inflammatory response.

Tendon healing is considered to occur in three overlapping phases: the inflammatory phase (1-1½ weeks) the reparative/proliferative healing phase (1-6 weeks) and remodelling phase (4- weeks)²². In the inflammatory phase increase in angiogenesis plays as a key step in tissue regulation via providing oxygen and nutrients to the injured side. HIF1 α is also recognised to control key aspects of inflammation, particularly leucocyte recruitment and subsequent cytokine production¹⁰. After preparation in the inflammatory phase, the reparative phase comes where the fibroblast takes the major role in tendon healing. In this phase naturally the HIF1 α decreases because HIF1 α shifts the cell metabolism from oxidative glycoxidation to glycolysis. If HIF1 α persists in high concentrations, because of the build-up reactive oxygen species, cell death could be seen and thus could cause impairment of biomechanical properties²³.

Altogether the facts indicate that environmental, also probably species dependent conditions and particular co-stimuli, are necessary to provoke pro- or anti-apoptotic effects of TNF α in the tendon²⁴. Tendon healing is probably influenced by the presence of proinflammatory cytokines such as TNF α . However tendon degeneration is also associated with increased TNF α in many studies, because tenocytes are highly activated by TNF α ²⁵. High levels of TNF α are secreted by activated macrophages and stimulate the downstream production of other pro-inflammatory cytokines such as interleukins 1, 6 and 8 and granulocyte-macrophage colony-stimulating factor by syn-

Figure 4. Changes in the mean TNF α levels by the time in both groups.

* represents the significant decrease of TNF α levels at 3rd week in both diclofenac and control group and † represents the significant difference in the TN α at 6th week.

ovial tissue via TNFR1 receptors. Also TNF α has TNFR2 receptors which modulate cell growth, differentiation and tissue repair⁹. The peak in the first week shows us the inflammatory phase of tendon healing. Rapid decrease at 3rd week and steady state at 6th week corresponds to reparative phase and may be a clue for tissue repair and cell growth modulation effect of TNF α .

In the diclofenac group, the HIF1 α levels at the first week were higher than the control group. Also, because HIF1 α up-regulates VEGF production, vascular proliferation was also high in diclofenac group at 1st week¹³. However there was a rapid decrease at 3rd week in the diclofenac group. Because diclofenac has a potent anti-inflammatory effect this block in the inflammation may cause feedback effect on HIF1 α and increase the HIF1 α levels at the first week. But, when the healing phase progresses to reparative phase the HIF1 α levels decrease rapidly. Diclofenac did not change TNF α levels because while diclofenac inhibited cyclooxygenases and directed eicosanoid synthesis from prostaglandins towards leukotrienes, TNF α produced by macrophages, monocytes, fibroblasts and CD4+ T helper cells and played as a key driver of pro-inflammatory cytokine and prostaglandin E2 synthesis²⁶. The interesting results were increased in TNF α levels from 3rd to 6th weeks. While HIF1 α was high in the first week, the cells could shift their metabolism to glycolysis and resulting in an increase of free oxygen radicals. The magnitude and duration of hypoxic microenvironment and free oxygen radicals determines whether cells become apoptotic or adapt and survive. HIF1 α is critical regulatory in this process²⁷. If microenvironment leads to necroptosis, which is a regulated form of necrosis, can trigger an innate immune response which could lead to increase in TNF α levels which could also impair the biomechanical properties of healed tendon²⁸.

There are many limitations of this study. First, simulated tendon tear was acute tear rather than chronic tear. In chronic tears, TNF α seemed to play a major role in the tendon degeneration²⁹. Second, we did not perform biomechanical testing. Although this is the main limitation of our study, in literature it is shown that inhibition of HIF prolyl 4 hydroxylases (a natural inhibitor of HIF1 α in the body) improved mechanical properties of the enthesis³⁰ and increased HIF1 α /VEGF neoangiogenesis impaired biomechanical properties of the tendon¹⁹. These findings are compatible with our findings. Thirdly we did not obtain samples at day 0 which could be more precise as the baseline levels. But this is the first study that evaluates the HIF1 α and TNF α levels in the acute setting of tendon repair and could put forth the possible effects of NSAIDs during healing via HIF1 α and TNF α .

As a conclusion, HIF1 α and TNF α both have a role in pro/antiinflammatory process. They both play a role especially in the inflammatory process however HIF1 α levels also correlate with vascular proliferation in tendon healing. The HIF1 α and TNF α levels were decreased as the time in normal tendon healing process. The diclofenac effects the levels of HIF1 α and TNF α levels during this process.

Compliance with ethical standards

Conflict of interest

No other relationships/conditions/circumstances that present potential conflict of interest.

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