Biochemical and Biophysical Research Communications 454 (2014) 301–307

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Effect of indomethacin and lactoferrin on human tenocyte proliferation and collagen formation *in vitro*

Yaonan Zhang [a,b], Xiao Wang [a], Yiwei Qiu [a], Jillian Cornish [c], Andrew J. Carr [a], Zhidao Xia [a,*]

[a] Centre for Nanohealth, College of Medicine, Swansea University, Singleton Park, Swansea, UK SA2 8PP
[b] Department of Orthopaedic, Beijing Hospital of Ministry of Public Health, Beijing, China 100730
[c] Department of Medicine, University of Auckland, Private Bag 92019, Auckland, New Zealand

ARTICLE INFO

Article history:
Received 6 October 2014
Available online 18 October 2014

Keywords:
Non-steroidal anti-inflammatory drugs (NSAIDs)
Indomethacin
Lactoferrin
Anabolic
Human tenocyte

ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in patients with injuries and inflammation of tendon and ligament, and as post-surgical analgesics. The aim of this study is to investigate the effect of indomethacin, a classic NSAID and its combinational effect with an anabolic agent of skeletal tissue, lactoferrin, on the proliferation and collagen formation of human tenocytes *in vitro*. A factorial experimental design was employed to study the dose-dependent effect of the combination of indomethacin and lactoferrin. The results showed that indomethacin at high concentration (100 μM) inhibited human tenocyte proliferation in culture medium with 1–10% fetal bovine serum (FBS) *in vitro*. Also, high dose of indomethacin inhibited the collagen formation of human tenocytes in 1% FBS culture medium. Lactoferrin at 50–100 μg/ml promoted human tenocyte survival in serum-free culture medium and enhanced proliferation and collagen synthesis of human tenocytes in 1% FBS culture medium. When 50–100 μg/ml lactoferrin was used in combination with 100–200 μM indomethacin, it partially rescued the inhibitory effects of indomethacin on human tenocyte proliferation, viability and collagen formation. To our knowledge, it is the first evidence that lactoferrin is anabolic to human tenocytes *in vitro* and reverses potential inhibitory effects of NSAIDs on human tenocytes.

1. Introduction

With a considerable morbidity, tendon injuries may cause pain and disability that last for several months [1]. However, the basic cell biology of tendons still has not been fully understood [2], and the management of tendon injury poses a substantial challenge for clinicians [3,4]. Conventionally, non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in patients with musculoskeletal conditions such as sports injuries, inflammation of tendons and ligaments, and also as a post-surgical analgesic [5]. Clinical studies indicate that NSAIDs reduce pain, tenderness and stiffness associated with acute soft tissue injuries [6]. NSAIDs are also used for managing other tendon related conditions such as chronic tendonitis, although histological studies have shown absence of acute inflammatory cells in some of these conditions [7,8]. A number of studies on bone, ligament, and tendon repair have evaluated the effects of NSAIDs in experimental animal tissue. It has been reported that NSAIDs may delay soft tissue healing with unknown mechanisms [9]. Currently, the effect of NSAIDs on tenocytes remains controversial, with both beneficial [10] and harmful [11,12] effects being described.

Indomethacin is the one of the earliest NSAIDs. It inhibits arachidonic acid metabolism via affecting lipoxygenase and cyclooxygenase and therefore is used as an anti-inflammatory drug [13]. It has been reported that indomethacin delays fracture healing [14], and obstructs bone formation [15]. Recently, studies indicate that indomethacin inhibits proliferation of primary tenocytes isolated from rat tendon [16], delays rat tendon healing [17] and impairs rotator cuff tendon-to-bone healing [12]. Controversially, there are also some reports suggesting that indomethacin could improve tendon healing in rats [10]. Therefore, it is of great interest to study the effect of indomethacin on tenocytes, and whether any negative effects can be reversed or prevented.

Lactoferrin is an iron-binding glycoprotein which is present in epithelial secretions, breast milk, and the secondary granules of neutrophils [18]. Bovine and human lactoferrin have been demonstrated to be anabolic to bone and cartilage tissue, stimulating osteoblast and chondrocyte proliferation, as well as increasing bone formation both *in vitro* [19,20] and *in vivo* [21]. However, there is no report of the effect of lactoferrin on human tenocytes.

* Corresponding author at: Centre for Nanohealth, College of Medicine, Swansea University, Singleton Park, Swansea, UK SA2 8PP.
E-mail address: z.xia@swansea.ac.uk (Z. Xia).

http://dx.doi.org/10.1016/j.bbrc.2014.10.061
0006-291X/

The aim of our present study is to investigate the effect of the classic NSAID indomethacin, and its combinational effect with lactoferrin, the anabolic agent of skeletal tissue, on the proliferation and collagen formation of human tenocytes *in vitro*. We found that indomethacin at high concentration (100 μM) inhibited human tenocyte proliferation and collagen formation, whereas lactoferrin at 50–100 μg/ml has a promotion effect. Combinational administration of lactoferrin with indomethacin rescued the harmful effects caused by indomethacin. Hopefully, this study may shed light on the design of therapeutic strategy in clinic.

2. Materials and methods

2.1. Materials

Indomethacin was purchased from Sigma–Aldrich Company Ltd. (Gillingham, UK). Bovine lactoferrin was isolated from fresh skim milk by cation exchange chromatography and gel filtration as described earlier [22]. α-Minimal essential medium (α-MEM) powder were purchased from Invitrogen, Paisley UK; 0.2 μm filter for culture medium preparation was from PALL Lifer Science, PALL Corporation, UK. Filters to remove endotoxin from any solution used were purchased from Sartorius Stedim Biotech, Surrey, UK. Fetal bovine serum (FBS) was purchased from Biosera, East Sussex, UK, and trypsin-EDTA, penicillin and streptomycin from Lonza Wokingham Ltd, Berkshire, U.K. Tissue culture grade polystyrene flasks and plates were obtained from Greiner Bio-One Company in UK. Unless otherwise noted, all other reagents were of analytical grade from Sigma–Aldrich, Poole, U.K., or VWR International Ltd, Lutterworth, U.K.

2.2. Isolation and culture of tendon derived cells

Human tendon biopsies were obtained from consenting, non-smoking, male patients <30 years of age who were undergoing right anterior cruciate ligament (ACL) reconstruction with a hamstring graft at the Nuffield Orthopaedic Centre, Oxford. The protocols were approved by the Oxford Research Ethics Committee C (09/H0606/11). Participants provide their written informed consent to participate in this study. The tendon samples were collected within 1 h following the operation and were kept in 4 °C sterile α-MEM medium before processing.

The tenocyte isolation method was adapted and modified from Bi et al. [23]. In general, 2 cm tendon segments from the middle of the tendon biopsies were diced into 1 mm^3 pieces and were treated in 4 mg/ml dispase (Roche, Hertfordshire, U.K.) and 300 U/ml collagenase type II (Gibco, Invitrogen, Paisley, U.K.) solution in serum free α-MEM (avoiding deactivating of enzymes) at 37 °C incubator for 16 h. After enzymatic digestion, equal volumes of α-MEM medium, supplemented with 10% FBS, were added to quench the collagenase and filtered through cell strainers (70 μm nylon, BD falcon, BD bioscience, California, U.S.A.). The filtered cell suspension was centrifuged at 1500 rpm (380 g) for 5 min, and the supernatant was discarded. Fresh culture medium with 20% FBS was used to boost cell proliferation after enzymatic release and single-cell suspension was cultured in 75 cm^2 tissue culture (TC) flasks (BD falcon, BD bioscience, California, U.S.A.), at 5% CO_2 and 95% air at 37 °C. The cells were sub-passaged after 80% confluence and cultured in the same culture medium supplemented with 10% FBS from passage 1.

2.3. Effect of indomethacin and lactoferrin on human tenocyte proliferation

Passage 3 human tenocytes was seeded in 96 well plates (BD falcon, BD bioscience, Oxford UK) at a density of 5000 cells/well in 1–10% FBS α-MEM medium. Indomethacin at different concentrations (0, 0.01, 0.1, 1, 10 and 100 μM) or lactoferrin at different concentrations (0, 10, 20, 30, 50 and 100 μg/ml) was added to culture medium and cells were cultured for 7–14 days. Culture medium was changed every 2–3 days. Alamar Blue™ assays were performed to determine cell proliferation according to manufacturer's protocol at day 0, day 4, day 7 and day 14. In brief, 200 μl of 5% Alamar Blue™ (Biosource Europe, Nivelles, Belgium) was added in each well after removal of the culture medium. The cells were incubated at 5% CO_2, 37 °C for 2 h. The Alamar Blue™ solution was transferred to another plate with 5 wells of 5% Alamar Blue™ as blanks and the relative fluorescent unit (RFU) was measured with SoftMax Pro software using a SPECTRAmax GEMINI micro plate spectrofluorimeter (Molecular Devices, Berks, U.K.) at the excitation wavelength of 530 nm and the emission wavelength of 590 nm, with a cut off of 570 nm. The estimated cell number was calculated by using a standard curve created by incubating 5% Alamar Blue™ under the same conditions. The cell number was plotted against the RFU. This process was performed repeatedly with each microplate in order to calculate the cell number.

2.4. Effect of lactoferrin on human tenocyte survival in serum free culture

Passage 3 human tenocytes were seeded in 96 well plates at a density of 5000 cells/well in 0% FBS α-MEM medium and were fed with different concentration of lactoferrin (0, 10, 20, 30, 50 and 100 μg/ml) for 7 days. Culture medium was changed every 2–3 days. AlamarBlue™ assay was performed at day 0 and day 7.

2.5. Assessment of the combinational effect of indomethacin and lactoferrin

A full factorial experimental design was employed to study the effect of the combination of the indomethacin and lactoferrin at effective doses selected from the first two experiments. The culture condition was carried out in 1% FBS culture medium. Passage 3 human tenocytes were seeded in 48 well plates at a density of 1×10^4 cells per well in 1% FBS α-MEM medium. The cells were treated with different concentrations of indomethacin and lactoferrin as shown in figures.

A tiered outcome analysis approached assessing (1) human tenocyte number changes (promoting or inhibiting cell proliferation); (2) collagen synthesis; and (3) cell morphology. For morphologic observation, glass cover slips were added into extra wells with cells seeded at the same time and cultured in the same condition. All experiments were repeated three times with consistent results.

2.6. Cell viability assay

Human tenocytes were stained with a fluorescent Live/Dead Viability/Cytotoxicity Kit (Invitrogen, Paisley, UK) at day 7, following the manufacturer's instructions. This fluorescent kit was used for cell labeling, with green for live cells and red for dead cells. Samples were examined by a fluorescent microscope (Carl-Zeiss Axio,) without fixation.

2.7. Evaluation of collagen synthesis

The procedure of Sirius red staining was adapted and modified from Tullberg-Reinert and Jundt [24]. On day 14, the culture medium was removed and the cell layers were washed extensively with PBS before being fixed with 100 μl of Bouin's Solution (71% saturated picric acid, 24% of formalin and 5% 0.5 M acetic acid) for 1 h. After fixation, the cell layers were washed under tap water

for 15 min and were air dried before being stained with 100 μl 0.1% Sirius red (Raymond A. Lamb, U.K.) dissolved in saturated picric acid (w/v) for 1 h. After staining, the unbound dye was removed by washing 5 times with 200 μl 0.01 M HCl. The bound dye was dissolved with 200 μl 0.1 M NaOH at room temperature for 30 min. The dye solution was transferred to another 96 well plate and the optical density (O.D.) was measured with a Bio-Rad micro plate reader (Dynex technologies, Channel Islands, U.K.) at 570 nm against five blank wells of 0.1 M sodium hydroxide. For quantification of collagen, soluble rat-tail collagen type I (Sigma–Aldrich, U.K.) ranging from 3.3 μg to 100 μg was used to create standard curves for each assay.

2.8. Statistics

Data are presented as mean ± standard deviation. At least 3 replicates of each experiment were performed, and the results presented in the figures are representative of these. For each variable, effects across treatment groups were compared with one-way analysis of variance (ANOVA). If the overall difference was significant, multiple comparisons were performed between groups using Tukey's test. Differences are considered significant at a probability <0.05 or <0.01 on a two tailed test.

3. Results

3.1. Indomethacin inhibited human tenocyte proliferation at high doses

We first examined the effect of indomethacin at different concentration on proliferation of tenocytes cultured in 10% FBS medium, which is the normal growth medium for the proliferation of most cells. As shown in Fig. 1, no differences of cell numbers among different groups at day 1 were observed as detected by Alamar Blue assay. The cell numbers showed 4–5-fold increases over 7 days in most doses (0–10 μM, $p < 0.01$) in comparison with day 1 of the same group. However, in 100 μM indomethacin group, which reflects the recommended clinical high dose, there were no cell number increase over the 7 days' culture, and the cell number at day 7 was significantly lower than that in the control group ($p < 0.01$). This result indicates that indomethacin at high doses may inhibit the proliferation of human tenocyte.

3.2. Lactoferrin promoted cell survival in serum-free medium

As lactoferrin has been demonstrated to be anabolic to bone and cartilage tissues, we examined its effect on tenocytes' survival. We found lactoferrin promoted cell survival in serum-free medium. As shown in Fig. 2, serum withdrawal from the culture medium resulted in significant cell number decreases ($p < 0.01$) over the 7-day culture period in all groups in comparison with the cell number at day1, as detected by Alamar Blue assay. Although no differences were observed when adding lactoferrin at low doses (10–30 μg/ml), significantly promoted cell survival were found in groups with lactoferrin at high doses (50–100 μg/ml).

3.3. Lactoferrin revived the inhibitory effect of indomethacin on tenocyte proliferation and viability

Since lactoferrin (LF) promoted cell survival in serum-free medium, we tested its combinational effects with indomethacin (ID) first on tenocyte proliferation by AlamarBlue assay. As demonstrated in Fig. 3A, there were small but significant cell number increases over a 14-day culture in the control group (LF 0 μg/ml, ID 0 μM). In the groups treated with ID alone (100 and 200 μM), there were significant decreases in cell numbers over 14 days, whereas 8 and 10-fold significant increases in cell numbers were found in the groups treated with LF alone (50 and 100 μg/ml), in a dose dependent pattern. When combined 100 μM ID with LF at either 50 μg/ml or 100 μg/ml, cell number decreases were observed in the first week but followed by a significant increase at day 14, in comparison with the control group at the same time point as well as with day 0 in the same group. Combined 200 μM ID with LF at both 50 and 100 μg/ml resulted in small but significant cell number increases compared with the control group and day 0 in the same group. Also, the cell number increases in the ID-LF combination treatment groups were significantly higher than the group treated with 200 μM ID alone.

In addition, we examined tenocyte viability by a Live/Dead Viability/Cytotoxicity assay. As shown in Fig. 3B, compared with control group, only LF alone at 100 μg/ml showed significantly higher viability ($p < 0.05$) at day 7. However, tenocyte viability in the groups with LF treatment at both 50 and 100 μg/ml combined with 100 μM ID was significantly higher than in the 100 μM ID alone treatment group. Together, these results indicate that high dose of lactoferrin may revive the inhibitory effect of indomethacin on tenocyte proliferation and viability.

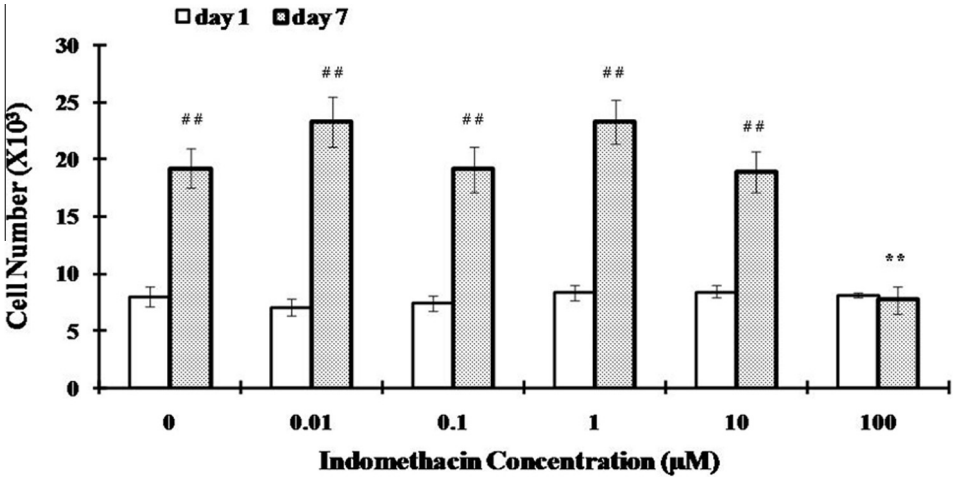

Fig. 1. Effect of indomethacin on human tenocyte proliferation in 10% FBS culture medium. **Compared with the control group at the same time point, $p < 0.01$; ##compared with day 1 in the same group, $p < 0.01$.

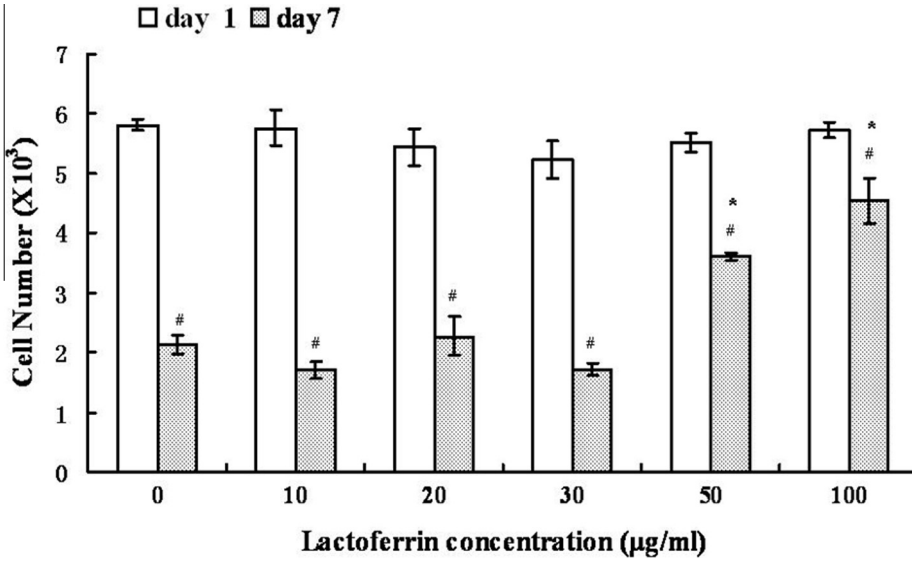

Fig. 2. Effect of lactoferrin on the survival of human tenocytes in serum-free (0% FBS) culture medium. *Compared with control group at the same time point, $p < 0.05$; #compared with day 1 in the same group, $p < 0.05$.

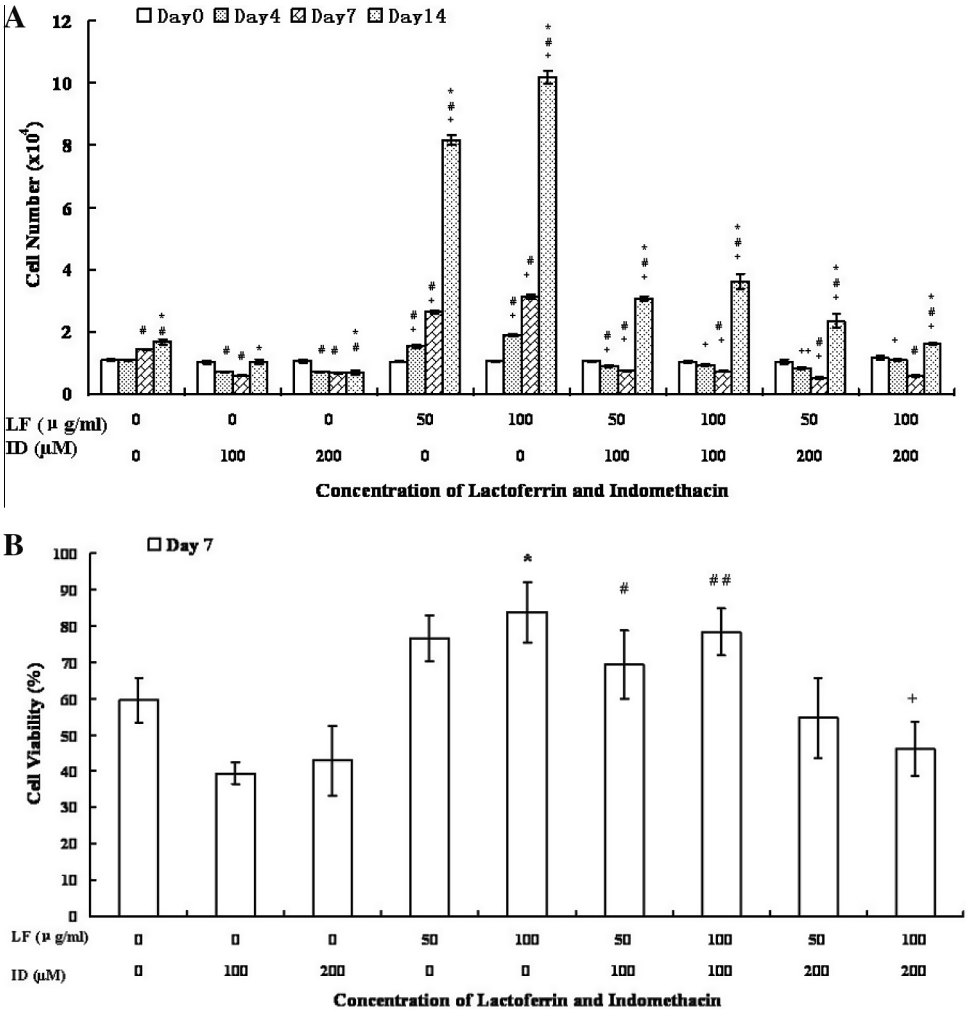

Fig. 3. Effect of indomethacin (ID) and lactoferrin (LF) and their combination on human tenocyte proliferation and viability. (A) Proliferation of human tenocyte with indicated treatments over 14 days in 1% FBS medium *in vitro*. *Compared with control group (ID 0 μM, LF 0 μg/ml) at the same time point, $p < 0.05$; #compared with day 0 in the same group, $p < 0.05$; +compared with the same doses, $p < 0.05$. (B) Viability of human tenocyte with indicated treatments in 1% FBS medium at day 7 *in vitro*. *Compared with control group (ID 0 μM, LF 0 μg/ml), $p < 0.05$; #compared with the same doses of ID without LF, $p < 0.05$; ##compared with the same doses of ID without LF, $p < 0.01$; +compared with the same doses of LF without ID, $p < 0.05$.

3.4. Lactoferrin rescued the inhibitory effect of indomethacin on tenocyte collagen formation

In addition, we measured the total collagen formation of tenocyte treated with indomethacin and lactoferrin over a 14-day culture period. As shown in Fig. 4, significant lower collagen formation were found in ID alone groups (100 and 200 μM), compared with control group ($p < 0.05$). LF alone groups (50 and 100 μg/ml) showed 6–8-fold increases of collagen formation over the control group ($p < 0.01$). When combined 100 μM ID with lactoferrin at either 50 or 100 μg/ml, the collagen formation was partly rescued, compared with the group treated with 100 μM ID alone ($p < 0.01$). However, LF treatment did not show any rescue effect on collagen formation when 200 μM ID was used.

4. Discussion

Local inflammation was involved in acute tendon injury, chronic degenerative injury and other tendon conditions. The function of inflammation is to remove injurious stimuli and initiate the process of tissue healing. The lasting pain caused by inflammation leads to the wide use of anti-inflammatory drugs, such as NSAIDs for acute and chronic inflammation. Indeed anti-inflammatory therapy reduces pain significantly and thus improves the quality of life for patients; however, whether it suppresses tendon healing processes or not is largely disregarded. Although the inhibitory effect of indomethacin on fracture healing has been reported for decades [25], there are only a handful of reports that have dealt with its effect on tendon healing [11,17,26,27].

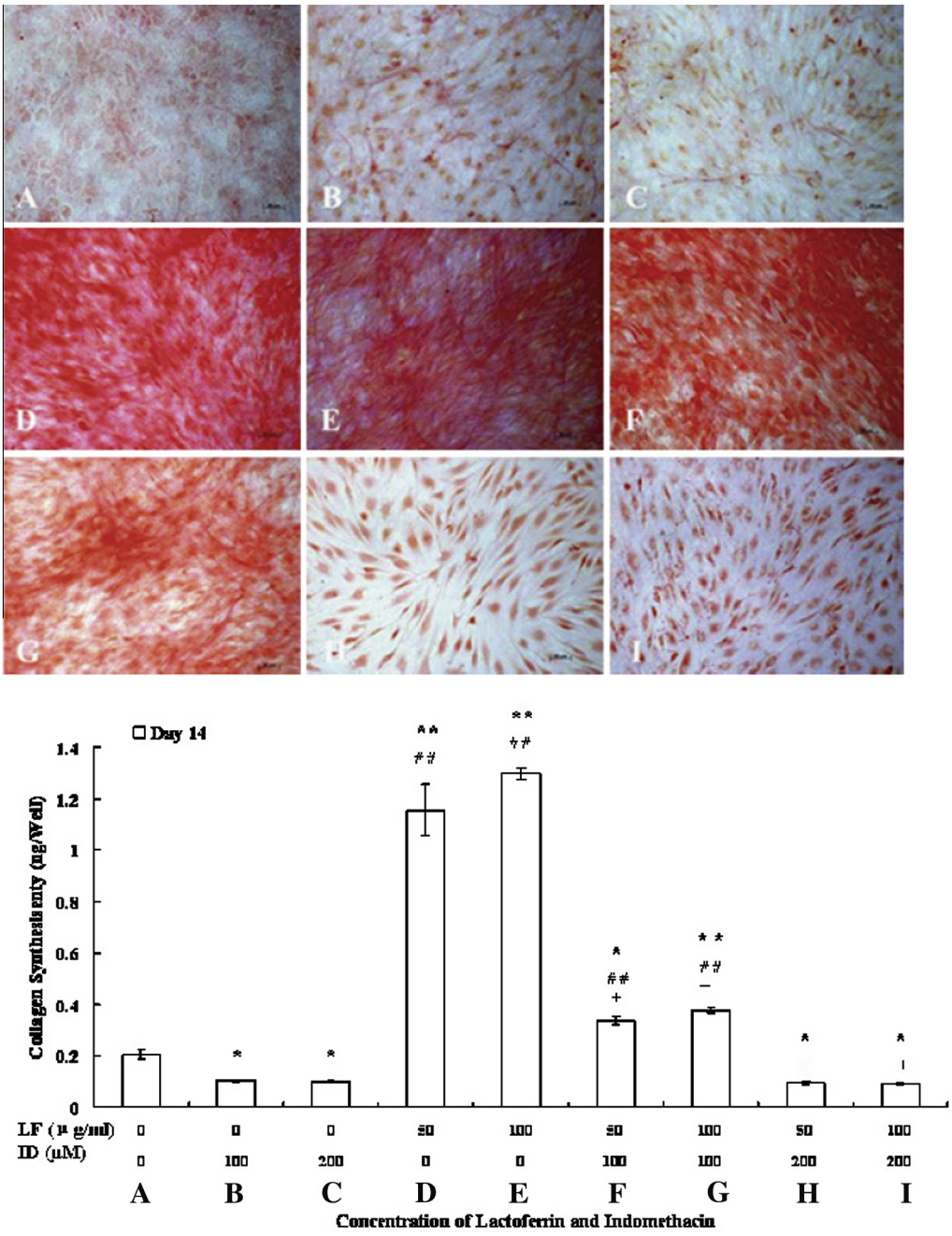

Fig. 4. Doses dependent effects of indomethacin (ID) and lactoferrin (LF) and their combination on human tenocyte collagen synthesis in 1% FBS medium at day 7 *in vitro*. *Compared with control group (ID 0 μM, LF 0 μg/ml), $p < 0.05$; ##compared with the same doses of ID without LF, $p < 0.01$; +compared with the same doses of LF without ID, $p < 0.05$.

Indomethacin inhibits arachidonic acid metabolism via affecting lipoxygenase and cyclo-oxygenase enzymes that participate in prostaglandin synthesis [28,29]. Prostaglandin E can act via kinase (ERK) signaling pathways to enhance osteoblast proliferation and differentiation [30]. Yet there is report showing that PGE2 inhibited tenocyte proliferation and collagen formation at 10 ng/mL and 100 ng/mL [31]. In this study we demonstrate that indomethacin suppresses human tenocyte proliferation and collagen formation at clinically-relevant high doses (100–200 μM) *in vitro*. At lower concentrations there were no significant effects of indomethacin on human tenocytes. Tenocytes synthesize prostaglandin [32] and it is not clear if the inhibitory effects at high doses are caused by regulation of prostaglandin formation. However, our results indicate that high doses of indomethacin may have potential negative effects on tenocytes proliferation.

The beneficial effect of anti-inflammatory drugs is well known, however, this effect should not be at a price of inhibition of tissue regeneration. Worst still, at the sites of injury, due to degeneration and chronic inflammation, there is likely to be conditions such as hypoxia, ischemia and harmful inflammatory factors releasing into the tissue. These local environments are not the ideal condition for tissue regeneration. Therefore, a combinational therapy of anti-inflammatory and anabolic drugs may have more potential to balance the local environment toward tissue regeneration.

Lactoferrin is a proven anabolic agent to bone and cartilage. However, to date there is no report of its effect on human tenocytes. This is the first study to show that lactoferrin is also an anabolic agent for human tenocytes, as it significantly promotes human tenocyte proliferation *in vitro* in culture medium with low and high dose of FBS supplement, as well as inhibits cell death in serum-free culture medium. More interestingly, we found that it reversed the inhibitive effects of indomethacin on human tenocyte proliferation. Factorial experimental design used in this study involves analyzing the effect of a combination of multiple factors and doses synergistically in order to select the most suitable combination. It was shown that 50–100 μg/ml lactoferrin can significantly reverse the suppression of 100 μM indomethacin on human tenocyte proliferation and total collagen formation. These findings demonstrate the benefits of combining NSAIDs and lactoferrin and suggest that there may be a combinational therapeutic benefit that not only reduces pain but also promotes tissue regeneration *in vivo*.

In this present study, we tested the effect of indomethacin and lactoferrin on human tenocyte proliferation and collagen formation *in vitro*. Yet further study evaluating the anabolic effect of lactoferrin on tenocyte and its deliver strategy using animal models would be needed. Also, the mechanism behind this remained largely unknown. Our further work may focus on exploring the cell signaling pathways involved, e.g. NF-κB, MAPK, PI3K-AKT pathways which control cell viability and proliferation, and also the pathways that involve in cell cycle and cell apoptosis.

In summary, here we reported that indomethacin at high doses (100–200 μM) inhibits human tenocyte proliferation and collagen formation, whereas lactoferrin at 50–100 μg/ml has a promotion effect. Combinational administration of lactoferrin with indomethacin rescued the harmful effects caused by indomethacin. These findings suggest that lactoferrin may have a role in reversing the likely inhibitory effects of NSAIDs on tendon repair and regeneration, which may shed light on the design of therapeutic strategy in clinic.

Conflict of interest statement

None declared.

References

[1] S.B. Weinfeld, Achilles tendon disorders, Med. Clin. North Am. 98 (2014) 331–338.
[2] D.S. Heckman, G.S. Gluck, S.G. Parekh, Tendon disorders of the foot and ankle, part 2: achilles tendon disorders, Am. J. Sports Med. 37 (2009) 1223–1234.
[3] P. Sharma, N. Maffulli, Tendon injury and tendinopathy: healing and repair, J. Bone Joint Surg. Am. 87 (2005) 187–202.
[4] B.M. Andres, G.A. Murrell, Treatment of tendinopathy: what works, what does not, and what is on the horizon, Clin. Orthop. Relat. Res. 466 (2008) 1539–1554.
[5] B. Su, J.P. O'Connor, NSAID therapy effects on healing of bone, tendon, and the enthesis, J. Appl. Physiol. 115 (2013) (1985) 892–899.
[6] H.G. Potter, J.A. Hannafin, R.M. Morwessel, E.F. DiCarlo, S.J. O'Brien, D.W. Altchek, Lateral epicondylitis: correlation of MR imaging, surgical, and histopathologic findings, Radiology 196 (1995) 43–46.
[7] Z.A. Duri, P.M. Aichroth, Patellar tendonitis: clinical and literature review, Knee Surg. Sports Traumatol. Arthrosc. 3 (1995) 95–100.
[8] K.M. Khan, N. Maffulli, Tendinopathy: an Achilles' heel for athletes and clinicians, Clin. J. Sport Med. 8 (1998) 151–154.
[9] L.C. Almekinders, Anti-inflammatory treatment of muscular injuries in sport. An update of recent studies, Sports Med. 28 (1999) 383–388.
[10] C. Forslund, B. Bylander, P. Aspenberg, Indomethacin and celecoxib improve tendon healing in rats, Acta Orthop. Scand. 74 (2003) 465–469.
[11] G.P. Riley, M. Cox, R.L. Harrall, S. Clements, B.L. Hazleman, Inhibition of tendon cell proliferation and matrix glycosaminoglycan synthesis by non-steroidal anti-inflammatory drugs in vitro, J. Hand Surg. Br. 26 (2001) 224–228.
[12] D.B. Cohen, S. Kawamura, J.R. Ehteshami, S.A. Rodeo, Indomethacin and celecoxib impair rotator cuff tendon-to-bone healing, Am. J. Sports Med. 34 (2006) 362–369.
[13] M.O. Hilario, M.T. Terreri, C.A. Len, Nonsteroidal anti-inflammatory drugs: cyclooxygenase 2 inhibitors, J. Pediatr. 82 (2006) S206–S212.
[14] S. Dimmen, L. Nordsletten, J.E. Madsen, Parecoxib and indomethacin delay early fracture healing: a study in rats, Clin. Orthop. Relat. Res. 467 (2009) 1992–1999.
[15] H. Saino, T. Matsuyama, J. Takada, T. Kaku, S. Ishii, Long-term treatment of indomethacin reduces vertebral bone mass and strength in ovariectomized rats, J. Bone Miner. Res. 12 (1997) 1844–1850.
[16] E. Mallick, N. Scutt, A. Scutt, C. Rolf, Passage and concentration-dependent effects of Indomethacin on tendon derived cells, J. Orthop. Surg. Res. 4 (2009) 9.
[17] S. Dimmen, L. Engebretsen, L. Nordsletten, J.E. Madsen, Negative effects of parecoxib and indomethacin on tendon healing: an experimental study in rats, Knee Surg. Sports Traumatol. Arthrosc. 17 (2009) 835–839.
[18] J.H. Brock, Lactoferrin – 50 years on, Biochem. Cell Biol. 90 (2012) 245–251.
[19] A. Grey, T. Banovic, Q. Zhu, M. Watson, K. Callon, K. Palmano, J. Ross, D. Naot, I.R. Reid, J. Cornish, The low-density lipoprotein receptor-related protein 1 is a mitogenic receptor for lactoferrin in osteoblastic cells, Mol. Endocrinol. 18 (2004) 2268–2278.
[20] A. Grey, Q. Zhu, M. Watson, K. Callon, J. Cornish, Lactoferrin potently inhibits osteoblast apoptosis, via an LRP1-independent pathway, Mol. Cell. Endocrinol. 251 (2006) 96–102.
[21] J. Cornish, K.E. Callon, D. Naot, K.P. Palmano, T. Banovic, U. Bava, M. Watson, J.M. Lin, P.C. Tong, Q. Chen, V.A. Chan, H.E. Reid, N. Fazzalari, H.M. Baker, E.N. Baker, N.W. Haggarty, A.B. Grey, I.R. Reid, Lactoferrin is a potent regulator of bone cell activity and increases bone formation in vivo, Endocrinology 145 (2004) 4366–4374.
[22] J. Cornish, A. Grey, K.E. Callon, D. Naot, B.L. Hill, C.Q. Lin, L.M. Balchin, I.R. Reid, Shared pathways of osteoblast mitogenesis induced by amylin, adrenomedullin, and IGF-1, Biochem. Biophys. Res. Commun. 318 (2004) 240–246.
[23] Y. Bi, D. Ehirchiou, T.M. Kilts, C.A. Inkson, M.C. Embree, W. Sonoyama, L. Li, A.I. Leet, B.M. Seo, L. Zhang, S. Shi, M.F. Young, Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche, Nat. Med. 13 (2007) 1219–1227.
[24] H. Tullberg-Reinert, G. Jundt, In situ measurement of collagen synthesis by human bone cells with a sirius red-based colorimetric microassay: effects of transforming growth factor beta2 and ascorbic acid 2-phosphate, Histochem. Cell Biol. 112 (1999) 271–276.
[25] E. Sudmann, E. Dregelid, A. Bessesen, J. Morland, Inhibition of fracture healing by indomethacin in rats, Eur. J. Clin. Invest. 9 (1979) 333–339.
[26] C.A. Carlstedt, K. Madsen, T. Wredmark, The influence of indomethacin on biomechanical and biochemical properties of the plantaris longus tendon in the rabbit, Arch. Orthop. Trauma. Surg. 106 (1987) 157–160.
[27] J. Thomas, D. Taylor, R. Crowell, D. Assor, The effect of indomethacin on Achilles tendon healing in rabbits, Clin. Orthop. Relat. Res. (1991) 308–311.
[28] P. Uotila, J. Mannisto, N. Simberg, K. Hartiala, Indomethacin inhibits arachidonic acid metabolism via lipoxygenase and cyclo-oxygenase in hamster isolated lungs, Prostaglandins Med. 7 (1981) 591–599.
[29] C.A. Carlstedt, K. Madsen, T. Wredmark, The influence of indomethacin on collagen synthesis during tendon healing in the rabbit, Prostaglandins 32 (1986) 353–358.
[30] Y. Hakeda, T. Hotta, N. Kurihara, E. Ikeda, N. Maeda, Y. Yagyu, M. Kumegawa, Prostaglandin E1 and F2 alpha stimulate differentiation and proliferation,

respectively, of clonal osteoblastic MC3T3-E1 cells by different second messengers in vitro, Endocrinology 121 (1987) 1966–1974.
[31] F. Cilli, M. Khan, F. Fu, J.H. Wang, Prostaglandin E2 affects proliferation and collagen synthesis by human patellar tendon fibroblasts, Clin. J. Sport Med. 14 (2004) 232–236.
[32] J.H. Wang, F. Jia, G. Yang, S. Yang, B.H. Campbell, D. Stone, S.L. Woo, Cyclic mechanical stretching of human tendon fibroblasts increases the production of prostaglandin E2 and levels of cyclooxygenase expression: a novel in vitro model study, Connect. Tissue Res. 44 (2003) 128–133.