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The effects of high glucose condition on rat tenocytes *in vitro* and rat Achilles tendon *in vivo*

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Objectives

The aim of this study was to investigate the effect of hyperglycaemia on oxidative stress markers and inflammatory and matrix gene expression within tendons of normal and diabetic rats and to give insights into the processes involved in tendinopathy.

Methods

Using tenocytes from normal Sprague-Dawley rats, cultured both in control and high glucose conditions, reactive oxygen species (ROS) production, cell proliferation, messenger RNA (mRNA) expression of NADPH oxidase (NOX) 1 and 4, interleukin-6 (IL-6), matrix metalloproteinase (MMP)-2, tissue inhibitors of matrix metalloproteinase (TIMP)-1 and -2 and type I and III collagens were determined after 48 and 72 hours *in vitro*. In an *in vivo* study, using diabetic rats and controls, NOX1 and 4 expressions in Achilles tendon were also determined.

Results

In tenocyte cultures grown under high glucose conditions, gene expressions of NOX1, MMP-2, TIMP-1 and -2 after 48 and 72 hours, NOX4 after 48 hours and IL-6, type III collagen and TIMP-2 after 72 hours were significantly higher than those in control cultures grown under control glucose conditions. Type I collagen expression was significantly lower after 72 hours. ROS accumulation was significantly higher after 48 hours, and cell proliferation after 48 and 72 hours was significantly lower in high glucose than in control glucose conditions. In the diabetic rat model, NOX1 expression within the Achilles tendon was also significantly increased.

Conclusion

This study suggests that high glucose conditions upregulate the expression of mRNA for NOX1 and IL-6 and the production of ROS. Moreover, high glucose conditions induce an abnormal tendon matrix expression pattern of type I collagen and a decrease in the proliferation of rat tenocytes.

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Keywords: High glucose, Oxidative stress, Tendinitis

Article focus

- This study focused on the effect on rat tenocytes under high glucose conditions *in vitro* and *in vivo*.
- How does high glucose affect oxidative stress in tenocytes?
- How does high glucose affect tendon matrix and inflammatory gene expression?

Key messages

- High glucose conditions induce oxidative stress, in the form of reactive oxygen

- species production through NADPH oxidase (NOX) upregulation in tenocytes.
- Oxidative stress induced by high glucose conditions may be one of the causes of tendon degeneration and inflammation.
- Inhibiting oxidative stress could be a target of the treatment for diabetes mellitus-related tendinitis or tendinopathy.

Strengths and limitations

- The *in vitro* glucose concentrations used in this study did not reflect the *in vivo* environment.

- The potential effects of NOX inhibitors on tenocytes were not examined.
- The mechanical properties of the diabetic rat Achilles tendon were not investigated.
- Hyperglycaemia did not appear to affect Achilles tendon structure until at least four weeks.

Introduction

Musculoskeletal disorders such as tendonitis,¹ Dupuytren's disease,² carpal tunnel syndrome,³ adhesive capsulitis,⁴ calcific tendinopathy,⁵ stiffness⁶ and frozen shoulder^{7,8} can be observed in patients with diabetes mellitus (DM). *In vivo* studies, investigating the effects of hyperglycaemia, and using histological and biomechanical parameters, have shown impaired tendon-bone healing in a rat model of rotator cuff tears.⁹

Oxidative stress induced by hyperglycaemia has been reported to cause tissue damage and organ dysfunction.¹⁰ Chronic inflammation, caused by oxidative stress, may also contribute to the development of other diabetic complications such as atherosclerosis and cardiovascular disease, as well as in other disease states such as malnutrition, anaemia and hyperparathyroidism.¹⁰ In hyperglycaemic states, oxidative stress is triggered by reactive oxygen species (ROS) and controlled by antioxidant enzymes such as superoxide dismutase and catalase.¹¹ Some pathways that produce ROS have relevance to the hypermetabolism of polyol,¹² the accumulation of advanced glycation end-products (AGEs)¹³ and the over-expression of a receptor for AGEs,¹⁴ the increase in superoxide production by the mitochondrial electron transfer system¹⁵ and the activation of NADPH oxidase (NOX).¹⁶ High glucose levels have been shown to stimulate ROS production through protein kinase C-dependent activation of NOX in cultured aortic smooth muscle cells and endothelial cells.¹⁶

Few studies have examined the molecular mechanisms underlying tendon disorders in the musculoskeletal conditions associated with DM. We hypothesize that hyperglycaemic conditions induce oxidative stress and subsequent inflammation and degeneration within tendons. This study investigates the effect of hyperglycaemia on oxidative stress markers and inflammatory and matrix gene expression within tendons.

Materials and Methods

All animal procedures were performed with the approval and guidance of the Animal Care and Use Committee of our institution.

***In vitro* experiments: cell preparation.** Achilles tendons were excised from 15 healthy male Sprague-Dawley rats of seven to eight weeks age.¹⁷ Tendons were washed twice with phosphate-buffered saline and cut into small pieces measuring approximately 1.0 mm³. Several pieces were placed on a culture plate. After five minutes of air

drying for enhanced adherence, using Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, Missouri) supplemented with 10% foetal bovine serum, 100 µg/mL streptomycin and 100 U/mL penicillin was added. For all experiments, cells were incubated in DMEM with two different glucose concentrations according to a previous report:¹⁷ 12 Mm (mmol/l) (control glucose group) and 33 Mm (high glucose group). The culture medium was exchanged every 24 hours to maintain the glucose concentration (n = 15 per group).

***In vitro* experiments: detection of ROS accumulation.** In total, 1×10^5 cells were seeded in 2 ml of DMEM in each well of 12-well plates and incubated with control or high glucose conditions at 5% CO₂ and 37°C until detection of ROS. The accumulation of ROS in cells was detected by using the Total ROS/Superoxide Detection Kit (Enzo Life Sciences, Farmingdale, New York) after 48 hours and 72 hours, according to the manufacturer's directions, and the nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI) (which stains nuclei specifically by binding to AT-rich regions in DNA). ROS accumulation was analyzed under a BZ-8000 confocal laser microscope (Keyence, Osaka, Japan) using a fluorescein isothiocyanate barrier filter. For quantitative analysis of ROS accumulation, fluorescence intensity was calculated by ImageJ (US National Institutes of Health, Bethesda, Maryland) and normalized to cell number as determined by 4',6-diamidino-2-phenylindole (DAPI) in five randomly selected fields (n = 15 per group).

***In vitro* experiments: quantitative real-time polymerase chain reaction (PCR).** Total RNA was extracted from the cell cultures, and incubated both in control or high glucose conditions for 48 hours and 72 hours using an RNeasy Mini Kit (Qiagen, Valencia, California). Total RNA was then reverse transcribed into single-stranded complementary (c)DNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, California). Real-time PCR was performed on the cDNA samples, in duplicate, on an Applied Biosystems 7900HT fast real-time PCR system and using SYBR Green reagents (Applied Biosystems) to analyze the messenger RNA (mRNA) levels of NOX1, NOX4, interleukin (IL)-6, type I and type III collagens, matrix metalloproteinase-2 (MMP-2) and tissue inhibitors of matrix metalloproteinase (TIMP)-1 and -2. (Table I) Results were normalized to the mRNA levels of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase and were expressed relative to their levels in control culture using the $2^{(-\Delta\Delta CT)}$ method (n = 15 per group).¹⁸

***In vitro* experiments: cell proliferation assays.** A total of 5000 cells were seeded in 100 µl of DMEM in each well of two 96-well plates and were incubated in control or high glucose conditions at 5% CO₂ and 37°C. At 48 hours and 72 hours, cell proliferation was measured by a water-soluble tetrazolium salt (WST) assay using Cell Counting

Table 1. Primer sequences used for polymerase chain reaction

Gene	Oligonucleotide sequence
NOX1	Forward 5' GTGGCTTTGGTTCTCATGGT 3' Reverse 5' TGAGGACTCCTGCAACTCCT 3'
NOX4	Forward 5' GGGCCTAGGATTGTGTTGA 3' Reverse 5' CTGAGAAGTTCAGGGCGTTC 3'
Type I collagen	Forward 5' TGGAGACAGGTCAGACCTG 3' Reverse 5' TATTCGATGACTGTCTTGCC 3'
Type III collagen	Forward 5' TAAAGGGTGAACGGGGCAGT 3' Reverse 5' ACGTCCCCATTATGGCCAC 3'
MMP-2	Forward 5' GGAAGCATCAAATCGGACTG 3' Reverse 5' GGGCGGGAGAAAGTAGCA 3'
TIMP-1	Forward 5' ATAGTGCTGGCTGTGGGGTGTG 3' Reverse 5' TGATCGCTCTGGTAGCCCTTCTC 3'
TIMP-2	Forward 5' GGACACGCTTAGCATCACCCAGA 3' Reverse 5' GTCCATCCAGAGGCACTCATCC 3'
IL-6	Forward 5' GGTCTTCTGGAGTTCGGTTTC 3' Reverse 5' GGCTTGGTCTTAGCCACTC 3'
GAPDH	Forward 5' GGTGGTCTCCTCTGACTTCAACA 3' Reverse 5' GTTGCTGTAGCCAAATTCGTTG 3'

Kit-8 (Dojindo, Kumamoto, Japan).¹⁹ Then, 10 µl of WST was added to each well and cultures were incubated for an additional three hours at 5% CO₂ and 37°C prior to spectrophotometric evaluation. The conversion of WST to formazan was spectrophotometrically measured at 450 nm. Total cell proliferation for each treatment condition was expressed as an n-fold difference from the control glucose group at each corresponding timepoint, and the optical density of cells in the control glucose group was set to one, as a reference point. To account for the potential impact of hyperosmolarity on tendon cell proliferation, parallel cultures were incubated in DMEM supplemented with mannitol at 12 mM or 33 mM (n = 15 per group).

In vivo animal experiment: type I diabetic rat model. To induce DM, seven eight-week-old healthy male Sprague-Dawley rats, with a mean weight of 296 g SD 3.66, were injected with a single intravenous dose of streptozotocin (STZ; 65 mg/kg body weight; Sigma-Aldrich) dissolved in sodium citrate buffer (pH 4.5). The ten control rats received citrate buffer by an intravenous injection.^{9,20} Following the injections, all animals were housed in standard cages with unrestricted food, water and activity. The animals were monitored according to a standardized protocol.²¹ All STZ-injected rats became diabetic. Their mean blood glucose sugar level was 413 mg/ml SD 7.78 (23 mM) in DM rats and 116 mg/ml SD 5.57 (6.4 mM) in control rats. The animals were sacrificed at six weeks after the STZ injection according to Nouruzian et al's method.²² Achilles tendons were harvested and stored at -80°C for further analysis. The right Achilles tendon was used for immunohistological evaluation and the left was used for quantitative real-time PCR.

In vivo animal experiment: quantitative real-time PCR. The Achilles tendons were cut into small pieces, carefully isolated from connective tissue contaminants and minced. Isolated Achilles tendons were enzymatically dissociated with type II collagenase (Worthington Biochemical Corporation, Lakewood, New Jersey) and prepared for RNA isolation.²³ Total RNA was extracted using a RNeasy Mini Kit. Reverse transcription into single-stranded cDNA and real-time PCR was performed as previously described. We evaluated NOX1 and 4 expression in the Achilles tendons of both control and diabetic rats.

The seven left tendons of diabetic rats and ten from the control rats were used for quantitative real-time PCR.

In vivo animal experiment: Achilles tendon histology and immunohistochemistry for NOX analysis. Frozen, long-axis sections of Achilles tendons embedded in Optimal Cutting Temperature compound (Sakura Finetek USA Inc., Torrance, California) were sequentially sectioned into 7 µm thick specimens and fixed using 10% phosphate-buffered paraformaldehyde at room temperature for 15 minutes. Histological evaluation of fibre structure, fibre arrangement, nuclear morphology and zonal variations in tendon cellularity was performed using haematoxylin and eosin (H&E) staining.²⁴ Each variable was scored between 0 and 3, with 0 being normal, 1 slightly abnormal, 2 abnormal and 3 markedly abnormal. The grading of H&E-stained sections from Achilles tendon samples was performed in five randomly selected optical fields in each histological section. Each field was evaluated by two blinded investigators (FT and TK). For the immunohistochemical evaluation of NOX expression, anti-NOX1 and anti-NOX4 antibodies (Abcam, Cambridge, UK) were used. Sections were incubated with proteinase for ten minutes, treated with 3% hydrogen peroxide (Wako Pure Chemical Industries, Osaka, Japan) to block endogenous peroxidase activity and incubated with anti-NOX1 or anti-NOX4 antibodies (1:100 for both) at 4°C overnight. Sections were then incubated with a peroxidase-labelled immunoglobulin antibody (Nichirei Bioscience, Tokyo, Japan) at room temperature for 30 minutes. The signal (NOX1 and NOX4) was detected by the formation of a brown colour following incubation with the peroxidase substrate 3,3'-diaminobenzidine (Nichirei Bioscience). Sections were then counterstained with haematoxylin and microscopically examined. For semi-quantitative analysis, the ratio of NOX-positive tendon cells per field was determined in five randomly selected fields for each tissue section. For each immunohistological analysis with H&E staining, NOX1 staining and NOX4 staining, the seven right tendons of diabetic rats and ten from control rats were used.

Statistical analysis. All data are expressed as means and SD. All statistical analyses of recorded data were performed using the Excel statistical software package (Ekuseru-Toukei 2015; Social Survey Research

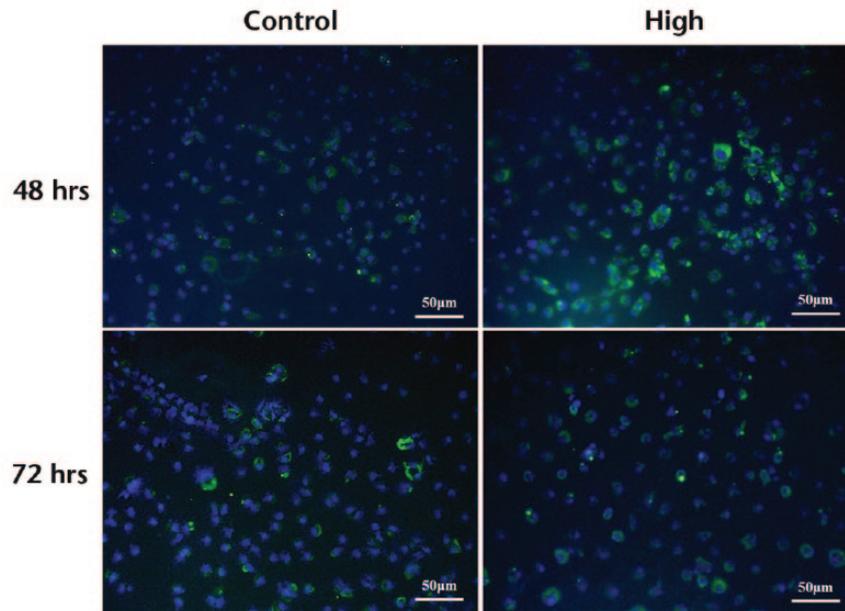


Fig. 1

Fluorescence staining showing reactive oxygen species (ROS) accumulation (green) in tenocytes and nuclei (4',6-diamidino-2-phenylindole) (blue). Increased ROS accumulation was observed in high glucose conditions compared with those in the control glucose conditions.

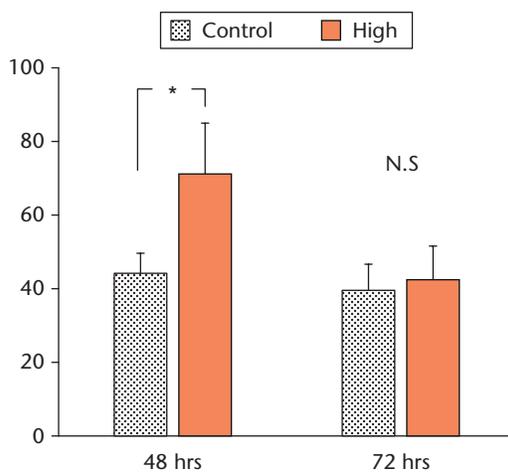


Fig. 2

Quantification of the accumulated of reactive oxygen species (ROS). The ROS accumulation was analyzed by fluorescence intensity normalized to cell number. The intensity was higher in the high glucose than in the control glucose conditions and significant difference was seen at 48 hours (* $p < 0.05$).

Information Co., Ltd., Tokyo, Japan). Comparisons between the control and high glucose conditions were made by analysis of variance, and the Mann-Whitney U test was used. Tendon pathological scores were analyzed using a Kruskal-Wallis test. A p -value < 0.05 was considered statistically significant.

Results

In vitro experiments: detection of ROS accumulation. Increased ROS accumulation was observed in cultures incubated in high glucose conditions compared with those incubated in control glucose conditions (Fig. 1).

At 48 hours after treatment, the intensity of fluorescence of ROS per cell incubated in control glucose and high glucose conditions were 44.2 ± 5.3 and 71.3 ± 13.6 , respectively (Fig. 2). At 72 hours after treatment, the ROS levels were 39.7 ± 6.9 and 42.4 ± 9.2 in control glucose and high glucose conditions, respectively (Fig. 2).

In vitro experiments: quantitative real-time PCR. The mRNA expression of NOX1 was significantly higher in tenocyte cultures incubated in high glucose conditions than in those incubated in the control glucose conditions both after 48 hours and after 72 hours (control 1.0 ± 0.46 , high 1.80 ± 0.64 , $p = 0.04$ at 48 hours; control 1.0 ± 0.23 , high 4.08 ± 0.84 , $p = 0.00039$ at 72 hours). Similarly, the mRNA expression of NOX4 was increased in cultures incubated in the high glucose conditions, with a significant difference observed after 48 hours, while there was no difference at 72 hours (control 1.0 ± 0.08 , high 1.27 ± 0.14 , $p = 0.01$ at 48 hours; control 1.0 ± 0.12 , high 1.58 ± 0.52 , $p = 0.27$ at 72 hours) (Fig. 3a). The mRNA levels of type I collagen and type III collagen did not show a significant difference between the groups after 48 hours (collagen I: control 1.0 ± 0.16 , high 1.14 ± 0.52 , $p = 0.48$; collagen III: control 1.0 ± 0.08 , high 1.0 ± 0.11 , $p = 0.95$). However, after 72 hours, the mRNA expression of type I collagen in cultures incubated in high glucose conditions was significantly lower and that of type III collagen was significantly higher in cultures incubated in high glucose conditions (collagen I: control 1.0 ± 0.12 , high 0.32 ± 0.27 , $p = 0.00094$; collagen III: control 1.0 ± 0.13 , high 3.27 ± 1.68 , $p = 0.002$) (Fig. 3a). Furthermore, MMP-2 and TIMP-1 mRNA levels were significantly higher in the high glucose group

after 48 hours and 72 hours (MMP-2: control 1.0 \pm 0.03, high 1.31 \pm 0.06, $p = 7.42 \times 10^{-95}$ at 48 hours; control 1.0 \pm 0.15, high 2.06 \pm 0.57, $p = 0.01$ at 72 hours; TIMP-1: control 1.0 \pm 0.02, high 1.75 \pm 0.22, $p = 4.38 \times 10^{-95}$ at 48 hours; control 1.0 \pm 0.28, high 11.6 \pm 0.70, $p = 1.33 \times 10^{-75}$ at 72 hours) (Fig. 3b). There was a significant increase in TIMP-2 and IL-6 expression in high glucose conditions after 72 hours compared with that in the control glucose conditions (TIMP-2: control 1.0 \pm 0.02, high 1.05 \pm 0.05, $p = 0.16$ at 48 hours; control 1.0 \pm 0.10, high 1.77 \pm 0.16, $p = 7.32 \times 10^{-55}$ at 72 hours; IL-6: control 1.0 \pm 0.80, high 1.70 \pm 0.22, $p = 0.22$ at 48 hours; control 1.0 \pm 0.11, high 2.01 \pm 0.51, $p = 0.023$ at 72 hours) (Fig. 3b).

In vitro experiments: cell proliferation assays. The WST assay of tendon cell proliferation showed that the proliferation of cells cultured in high glucose conditions was significantly lower than that of cultures in the control

glucose conditions after both 48 hours and 72 hours (control 1.0 \pm 0.10, high 0.69 \pm 0.02, $p = 0.005$ at 48 hours; control 1.0 \pm 0.03, high 0.54 \pm 0.06, $p = 0.005$ at 72 hours). Relative fold changes in proliferation are shown in Figure 4. In contrast, there were no significant differences in proliferation between cultures incubated in control (12 mM) and high (33 mM) mannitol concentration after 48 hours and 72 hours (control 1.0 \pm 0.02, high 0.99 \pm 0.08, $p = 0.84$ at 48 hours; control 1.0 \pm 0.04, high 0.97 \pm 0.03, $p = 0.22$ at 72 hours).

In vivo animal experiments: Achilles tendon histology and immunohistochemistry for NOX analysis. Histological evaluation showed no significant difference was observed in fibre structure, fibre arrangement, rounding of the nuclei and regional variations in cellularity between control and diabetic Achilles tendons (Table II). In both the fibre structure and fibre arrangement, control and diabetic tendons showed similar near parallel collagen fibres orientation to

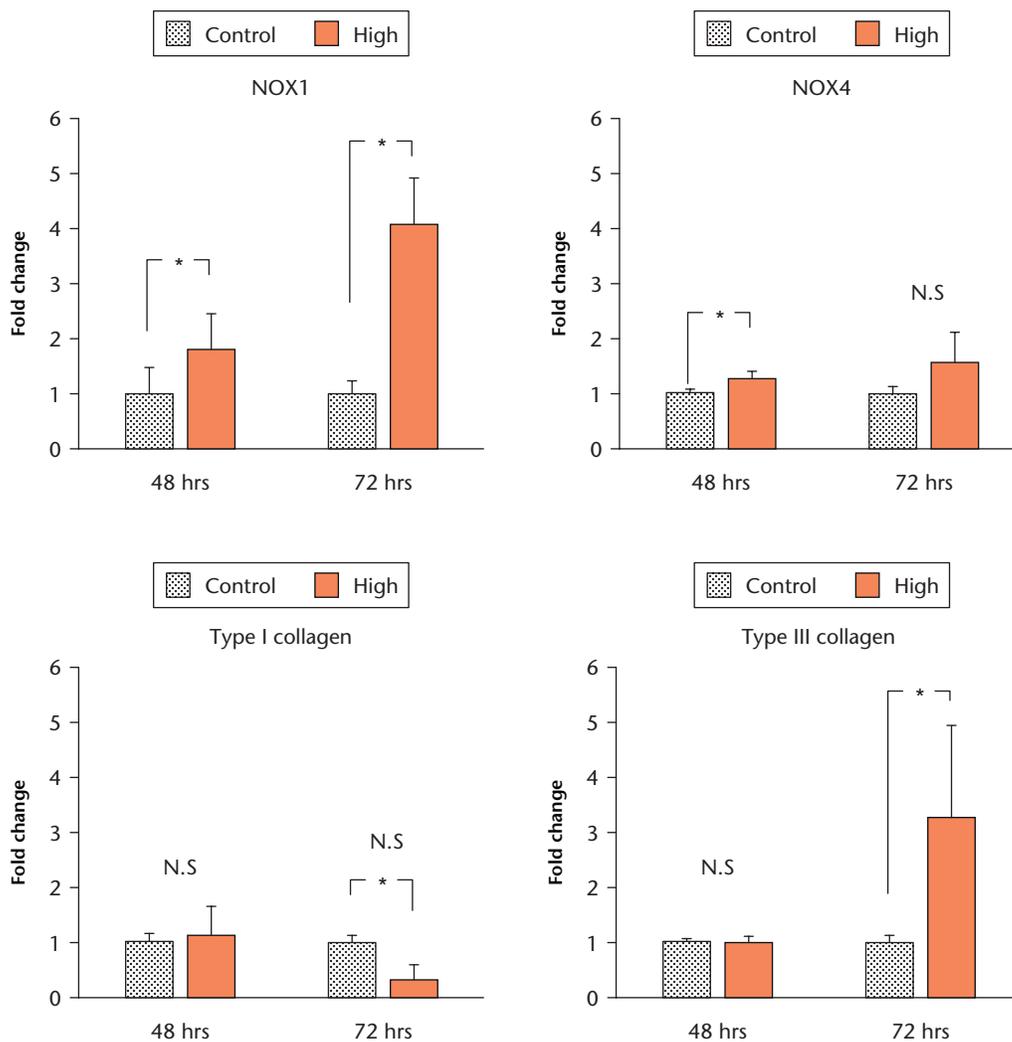


Fig. 3a

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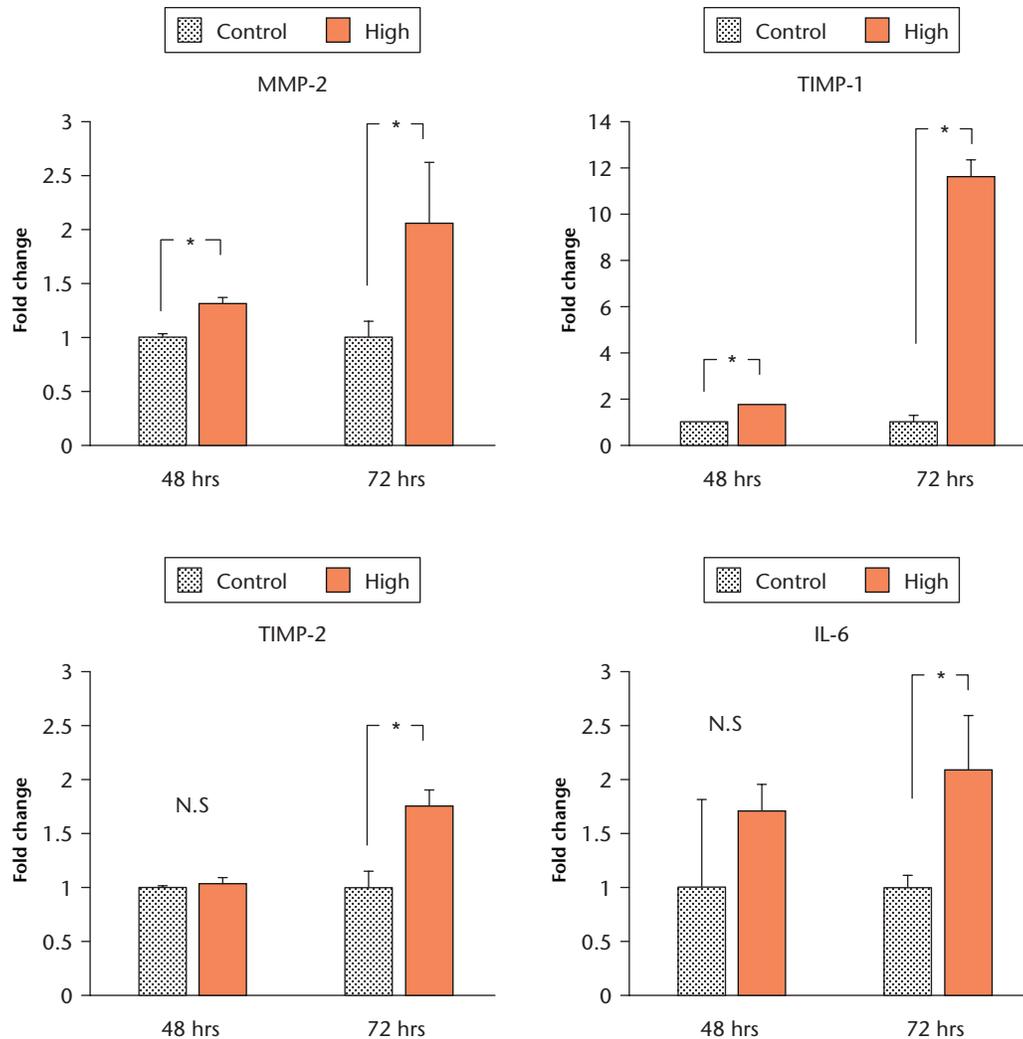


Fig. 3b

The messenger RNA (mRNA) expressions of NOX1 and NOX4 in the high glucose conditions were significantly higher than those in control glucose conditions at 48 hours. At 72 hours, the expression of NOX1 was significantly higher in the glucose conditions, while there was no significant difference in the expression of NOX4 between the groups. (* $p < 0.05$). There was no significant difference in the mRNA expression of type I and type III collagen between the two conditions at 48 hours. At 72 hours, in high glucose conditions, the expression of type I collagen was lower while the expression of type III collagen was higher than that in control glucose conditions (* $p < 0.05$); b) mRNA expressions of MMP-2 and TIMP-1 were significantly higher in high glucose conditions than those in control glucose conditions at 48 hours and 72 hours. There was a significant increase in TIMP-2 expression in high glucose conditions at 72 hours compared with that in the control glucose conditions (* $p < 0.05$). The mRNA expression of IL-6 was higher in high glucose than that in control glucose conditions at 48 hours and 72 hours, and the significant difference was observed at 72 hours (* $p < 0.05$).

each other (Fig. 5). Tenocytes within the control and diabetic tendons showed flattened or spindle shaped nuclei, arranged in rows between the collagen fibres, and few rounded nuclei were observed (Fig. 5). There were no regional variations in cellularity, either in control or diabetic tendons (Fig. 5). Immunohistochemical staining of the Achilles tendon at six weeks followed the STZ injection and NOX1 expression markedly increased within the tenocytes of the diabetic rats compared with the controls (Figs 6a and 6b). However, NOX4 was weakly expressed in both groups and showed no difference between the groups (Figs 6c and 6d). Using semi-quantitative analysis, the percentage of NOX1-positive cells was significantly

higher in the Achilles tendon of diabetic rats compared with the non-diabetic rats (control 12.3 \pm 4.64, diabetic 30.7 \pm 5.30, $p = 0.001$) (Fig. 7). There was no significant difference in the percentage of NOX4-positive cells between the groups (control 12.3 \pm 3.29, diabetic 13.3 \pm 4.23, $p = 0.71$) (Fig. 7).

In vivo animal experiments: quantitative real-time PCR. The mRNA expression of NOX, collagens, MMP-2, TIMP-2 and IL-6 in rat Achilles tendon was analyzed as an *in vivo* experiment. The expression of NOX1 in Achilles tendons was significantly higher in diabetic rats than in control rats (control 1.0 \pm 0.22, diabetic 1.59 \pm 0.34, $p = 0.028$). There was no significant difference in the NOX4

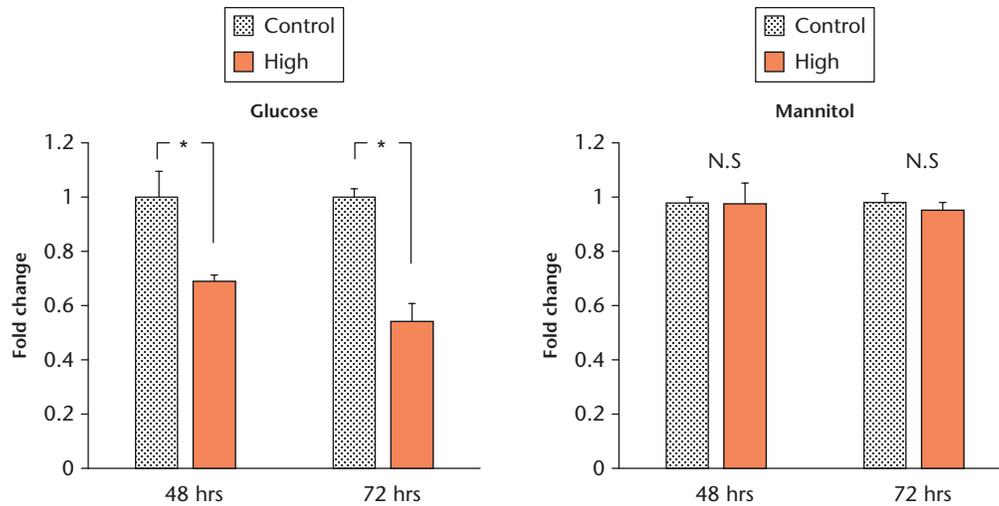


Fig. 4

Graphs showing relative fold changes in the proliferation of tenocytes in control and high glucose concentrations at 48 and 72 hours, along with similar concentrations of mannitol (* $p < 0.05$) (N.S., not significant).

Table II. Tendon pathological scores from haematoxylin and eosin staining. No significant difference was observed in fibre structure, fibre arrangement, nuclear morphology or zonal variations in cellularity between control and diabetic Achilles tendons

	Mean control (sd)	Mean diabetic (sd)	p-value*
Fibre structure	0.75 (1.04)	0.83 (0.72)	0.73
Fibre arrangement	0.75 (0.87)	1.00 (0.76)	0.51
Nuclear morphological changes (rounding)	0.50 (0.67)	0.63 (0.74)	0.76
Regional variations in cellularity	0.33 (0.65)	0.38 (0.52)	0.76

*Kruskal-Wallis test

n = 10 rats in the control group, n = 7 in the diabetic group

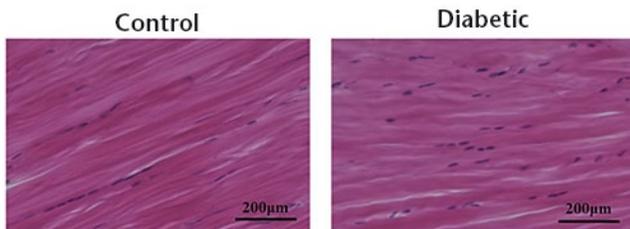


Fig 5

Achilles tendon histology. Haematoxylin and eosin staining of control and diabetic Achilles tendons harvested at six weeks following streptozotocin treatment. No obvious pathological difference between control and diabetic tendons was observed.

expression between control rats and diabetic rats (control 1.0 \pm 0.35, diabetic 1.29 \pm 0.66, $p = 0.47$) (Fig. 8a). The expressions of type I collagen (control 1.0 \pm 0.01, diabetic 1.24 \pm 0.02, $p = 0.01$), MMP-2 (control 1.0 \pm 0.47, diabetic 3.08 \pm 0.40, $p = 0.0005$), TIMP-2 (control 1.0 \pm 0.22, diabetic 2.79 \pm 0.23, $p = 2.90 \times 10^{-5}$) and IL-6 (control 1.0 \pm 0.47, diabetic 4.09 \pm 0.96, $p = 0.001$) were significantly higher in the diabetic rats than in the controls (Figs 8a and 8b), while expression of type III collagen did not show a significant difference (control 1.0 \pm 0.09, diabetic 1.04 \pm 0.21, $p = 0.81$) (Fig. 8a).

Discussion

DM has previously been suggested to increase susceptibility to tendinopathy.^{25,26} However, the molecular mechanisms underlying tendinopathy are unknown. A number of studies have demonstrated that hyperglycaemic conditions induce oxidative stress and cytokine production, leading to inflammation and tissue damage in various organs.²⁷⁻²⁹ Oxidative stress under experimental high glucose conditions causes an increase in ROS production.^{15,30} The production of ROS has been shown to lead tissue damage in various cell types.^{27,31,32} ROS are catalyzed by the multi-subunit enzyme NOX, which is located on the cell membrane.³³ NOX-derived ROS are essential modulators of signal transduction pathways that control key physiological activities such as cell growth, proliferation, migration, differentiation, apoptosis, immune responses and biochemical pathways.³⁴ However, under pathological conditions, the upregulation of tissue- and disease-specific NOX subtypes can cause overproduction of ROS.³⁴

To date, there are no reports about the role of NOX as a ROS-producing enzyme in the tenocyte.

A recent study showed that the expression of peroxiredoxin-5, which has antioxidant properties, is increased in tendinopathy, suggesting that oxidative stress is

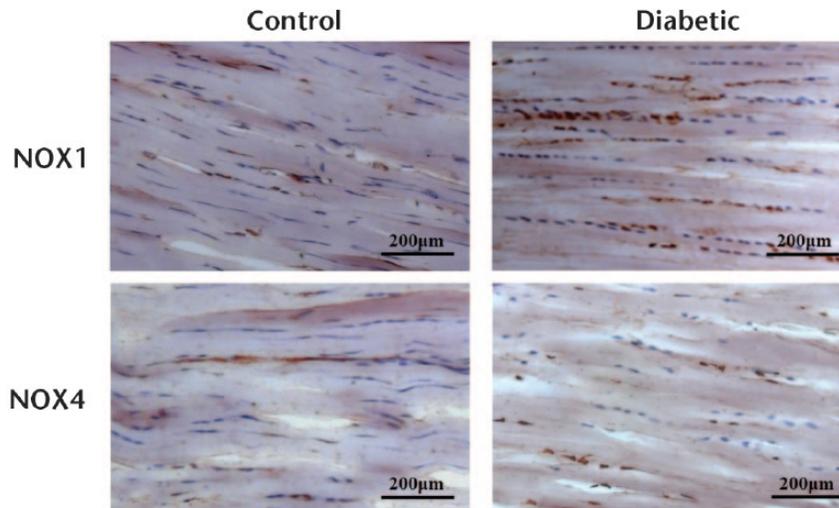


Fig. 6

Immunohistochemical staining for NADPH oxidase (NOX)1 and NOX4 expression in the Achilles tendon. The brown-stained cells were NOX-positive. Increased expression of NOX1 in Achilles tendon was observed in diabetic rats compared with control rats. There was no difference in the expression of NOX4 between diabetic and control rats.

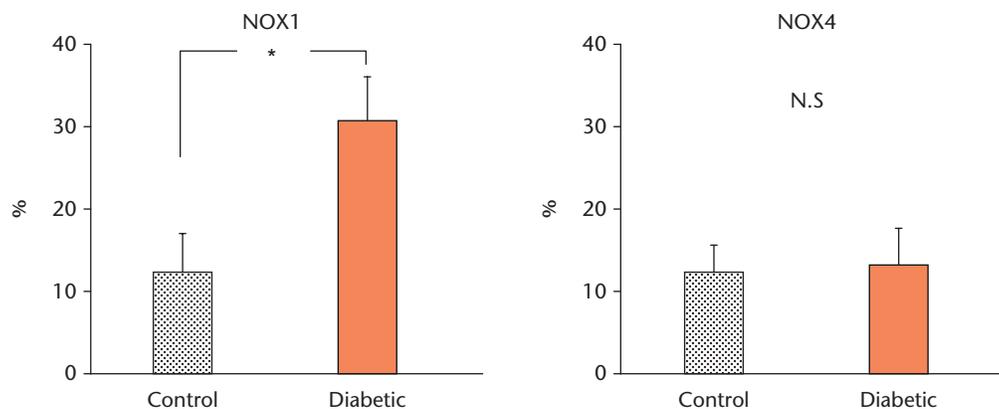


Fig. 7

Graphs showing the semiquantitative analysis of cells positive for NADPH oxidase (NOX)1 (left) and NOX4 (right). The ratio of NOX1-positive cells in Achilles tendon was significantly higher in the diabetic rat than in the control rat. No significant difference was observed between control and diabetic rats in the ratio of NOX4-positive cells (* $p < 0.05$).

involved in the pathogenesis of tendon degeneration.³⁵ Another study demonstrated that intracellular oxidative stress decreases type I collagen expression in the fibrocartilage layer of rotator cuff enthesis, leading to its degeneration.³⁶ In the present study, ROS production and NOX1 expression were increased in rat tenocytes under high glucose conditions. Moreover, our *in vivo* study using rat Achilles tendon showed increased NOX1 expression in tenocytes of diabetic rats. According to a study conducted on human aortic endothelial cells under high glucose conditions, NOX1 induced both inflammation and fibrosis.³⁷ The results of our study show that high glucose conditions upregulate the mRNA expression of NOX1 and the production of ROS at 48 hours. At 72 hours, ROS production did not show a significant difference between the groups. A possible explanation is that high glucose concentration decreased cell proliferation

and cell number which led to a decrease of ROS production after the longer time period.³⁸ Another possible explanation is that as a consequence of NOX4 up regulation, ROS production only increases during the early phases of high glucose stress. As another possibility, NOX4 upregulation at 48 hours only participated in ROS production in the early phase of high glucose stress.

Previous reports have shown that gene expressions of cytokines (IL-1 β , IL-1, IL-6, tumour necrosis factor (TNF)- α) are increased in the subacromial bursa in patients with rotator cuff disease.³⁹ This upregulation of inflammatory mediators is strong evidence that inflammation is a key process in tendinopathy.⁴⁰ A systematic review on cytokines in tendon disease showed that the expressions of IL-1 β , IL-6 and TNF- α in animal tendon injury models tend to increase from the early phase of tendon healing, whereas IL-6 was the only cytokine involved in human

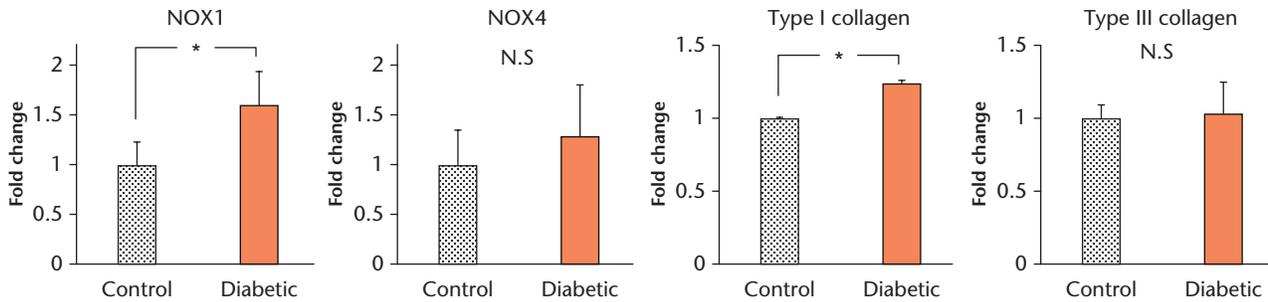


Fig. 8a

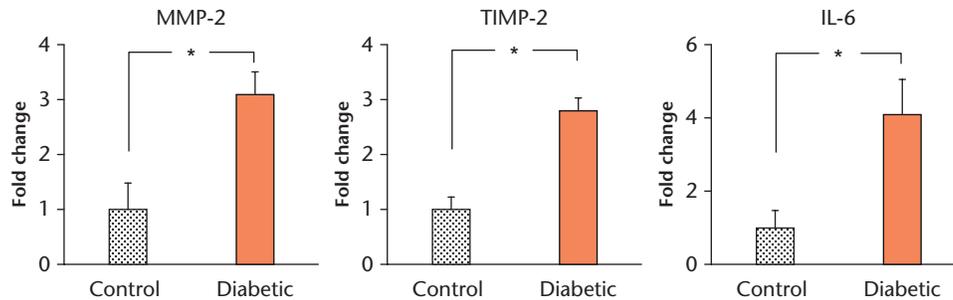


Fig. 8b

Graphs showing relative fold changes in the messenger RNA (mRNA) levels in tendon: a) mRNA expression of NADPH oxidase (NOX)1 in Achilles tendon was significantly higher in diabetic rats than in control rats. No significant difference between control and diabetic rats was seen in the expression of NOX4 (* $p < 0.05$). The expression of type I collagen was significantly higher in diabetic rats than in control rats. No significant difference was observed between diabetic and control rats in the expression of type III collagen (* $p < 0.05$); b) mRNA expression of matrix metalloproteinase (MMP)-2, tissue inhibitors of matrix metalloproteinase (TIMP)-2 and interleukin (IL)-6 in diabetic rat Achilles tendon was significantly higher than in the control.

tendon disease and found to be elevated in tendon tears.⁴¹ Further investigation is needed into the role of these cytokines in the development of tendon disease. In this study, the higher expression of IL-6 in tenocytes *in vitro* and Achilles tendon from diabetic rats was seen under high glucose conditions. These results indicate that high glucose conditions are associated with and might stimulate inflammatory processes within tendon.

The balance between expression of MMPs and TIMPs regulates normal tendon metabolic activity.⁴² During inflammation, MMPs cleave damaged interstitial collagen for remodelling, while TIMPs inhibit the overexpression of MMPs.⁴² High glucose has been reported to increase MMP-2 production in adventitial fibroblasts.⁴³ In the present study, high glucose conditions also increased the expression of MMP-2. TIMP-1 is not present in normal tendon but has been identified in the edges of torn supraspinatus tendon.⁴⁴ Our expression patterns of these cellular enzymes resembled those observed in acute tendon tears.

Approximately 90% of the collagen in normal tendon is type I, whereas type III collagen is expressed during inflammatory processes.⁴⁵ High glucose conditions have been found to inhibit type I collagen expression in human periodontal ligament fibroblast cultures.⁴⁶ Our results show that high glucose conditions lead to the decreased expression of type I collagen and the increased expression of type III collagen and these findings are compatible

with previous reports.^{45,46} In this study, diabetic Achilles tendons harvested at six weeks after induction of DM showed no histological difference compared with control tendons. Previous studies have not shown any significant difference in tendon histology in the diabetic rat model either in the acute onset of diabetes (one week) or in more chronic conditions (ten weeks) compared with controls.⁴⁷ The results from our study of acute phase reactions were similar. We observed that the expression of type I collagen, MMP-2 and TIMP-2 in the Achilles tendon of diabetic rats was higher than that of control rats. These results indicated that high glucose conditions might affect tendon matrix synthesis and turnover.

The effects of hyperglycaemia on cell proliferation in *in vitro* studies have been shown to be varied.^{48,49} Initially, glucose stimulates cell proliferation and thereafter inhibits the proliferation of rat peritoneal fibroblasts.⁴⁹ In other cell lines, such as human osteoblast-like cells MG63, high glucose conditions inhibit cell proliferation.⁴⁸ In the present study using control rat tenocytes, it was found that high glucose conditions decreased cell proliferation. A high concentration of mannitol had no effect on the proliferation of tenocytes. This shows that reduction of tenocyte proliferation was not influenced by hyperosmolarity. Moreover, in human tenocytes, oxidative stress by hydrogen peroxide has been demonstrated to induce apoptosis in high glucose conditions via bcl-2 mediated apoptosis through miR-28-5p and p53 upregulation.⁵⁰ Therefore,

hyperglycaemia might inhibit the ability to repair the damaged or degenerated tendon.

This study has several limitations. First, the *in vitro* glucose concentrations used in this study do not reflect the *in vivo* environment. The glucose concentrations in our experiments were considerably higher than the normal human plasma glucose level of 6 mM. The concentrations we chose were based on previous reports evaluating the effects of high glucose conditions on human and mouse endothelial and cardiac muscle cells.^{30,42,51} Secondly, the monolayer culture of tenocytes *in vitro* never reproduces true physiological conditions. However, previous studies have demonstrated that primary tenocytes maintained phenotypical stability until passage 5 when passaged in subconfluence.^{52,53} In the present experiments, cells were passaged at about 70% confluence and used by passage 3.^{19,53} Isolation of cells from the native tendon was performed according to previously reported and accepted methods.¹⁷ Thirdly, we did not investigate the biomechanical properties of the Achilles tendon of the diabetic rat due to low experimental numbers. It has been reported that the maximum tensile strength of the Achilles tendon in the STZ-treated animals is significantly decreased.⁵⁴ Further investigations are needed to explore the relevance of high glucose conditions on tendon biomechanical properties. Fourthly, the potential effects of NOX inhibitors on tenocytes were not examined. It has been previously reported that in DM, NOX1 mediates oxidative stress, inflammation and fibrosis and determines plaque size in atherosclerosis. NOX1 inhibition (by siRNA or GKT137831) is associated with reduced generation of ROS and attenuates DM-induced adhesion of inflammatory cells to the vascular wall, which is a key initiating step in the development of atherosclerosis.³⁷

In conclusion, high glucose concentrations upregulated the mRNA of NOX1, IL-6 expression and production of ROS in our experimental model. High glucose concentrations also induced abnormal tendon matrix expression of type I collagen and a decrease in proliferation of rat tenocytes under experimental conditions. These findings may be of importance in understanding the processes involved in tendinopathy.

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- T. Kokubu: Study design, Data analysis, Interpretation, Critically revising the paper.
- R. Kuroda: Study design, Data analysis, Interpretation, Critically revising the paper, Editorial contribution.

Conflict of Interest Statement

- None declared

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