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BIOMATERIALS

Evaluation of apocynin in vitro on high glucose-induced oxidative stress on tenocytes

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Aims

The purpose of this study was to evaluate the in vitro effects of apocynin, an inhibitor of nicotinamide adenine dinucleotide phosphate oxidase (NOX) and a downregulator of intracellular reactive oxygen species (ROS), on high glucose-induced oxidative stress on tenocytes.

Methods

Tenocytes from normal Sprague-Dawley rats were cultured in both control and high-glucose conditions. Apocynin was added at cell seeding, dividing the tenocytes into four groups: the control group; regular glucose with apocynin (RG apo+); high glucose with apocynin (HG apo+); and high glucose without apocynin (HG apo–). Reactive oxygen species production, cell proliferation, apoptosis and messenger RNA (mRNA) expression of NOX1 and 4, and interleukin-6 (IL-6) were determined in vitro.

Results

Expression of NOX1, NOX4, and IL-6 mRNA in the HG groups was significantly higher compared with that in the RG groups, and NOX1, NOX4, and IL-6 mRNA expression in the HG apo+ group was significantly lower compared with that in the HG apo– group. Cell proliferation in the RG apo+ group was significantly higher than in the control group and was also significantly higher in the HG apo+ group than in the HG apo– group. Both the ROS accumulation and the amounts of apoptotic cells in the HG groups were greater than those in the RG groups and were significantly less in the HG apo+ group than in the HG apo– group.

Conclusion

Apocynin reduced ROS production and cell death via NOX inhibition in high-glucose conditions. Apocynin is therefore a potential prodrug in the treatment of diabetic tendinopathy.

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Keywords: High glucose, Oxidative stress, Apocynin, NADPH oxidase, Diabetic tendinopathy

Article focus

- What is the antioxidant effect of apocynin on rat tenocytes under high-glucose conditions in vitro?
- How does apocynin affect cell viability and cell death under high-glucose conditions?
- How does apocynin affect inflammatory gene expression?

Key messages

- Apocynin could effectively reduce the levels of reactive oxygen species (ROS) by inhibiting the activity of nicotinamide adenine dinucleotide phosphate oxidase (NOX).
- Apocynin could exert anti-inflammatory effects by inhibiting oxidative stress.
- Apocynin is a potential prodrug in the treatment of diabetic tendinopathy.

Strengths and limitations

- This is the first report to evaluate the effects of apocynin on high glucose-induced oxidative stress on tenocytes.
- Further animal studies should be carried out to validate the effects of apocynin for oxidative stress in diabetic tenocytes.

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- There are several pathways regarding apoptosis under high-glucose conditions, and the mediator of the apoptotic signalling pathways was not investigated.

Introduction

Diabetes mellitus is associated with many musculoskeletal disorders including tendinitis, joint stiffness, tendon ruptures, adhesive capsulitis, and impaired tendon healing ability.[1-5] Longo et al[6] reported that patients with rotator cuff tears showed statistically significantly higher fasting plasma glucose levels within the normoglycaemic range than patients with meniscal tears. They concluded that increasing plasma glucose levels might be a risk factor for rotator cuff tears.[6] Reports suggest that musculoskeletal disorders of the hand and shoulder are more common in diabetic patients than in nondiabetic patients.[7] This can be attributed to the excessive generation of oxidative stress caused by hyperglycaemia.[8] High-glucose environments have previously been reported to increase intracellular reactive oxygen species (ROS),[9] which are the main bodies of oxidative stress. Increases in ROS cause damage to DNA, RNA, and proteins, as well as alterations in antioxidant enzyme levels which lead to cellular and tissue damage.[10,11] According to previous reports, the main source of ROS is nicotinamide adenine dinucleotide phosphate oxidase (NOX), and its activation increases ROS production.[12-14] Reactive oxygen species derived from NOX play a vital role in initiating and accelerating the progression of diabetic complications.[15] NOX contains regulatory phagocyte oxidase (phox) subunits $p67^{phox}$, $p47^{phox}$, $p22^{phox}$, $p40^{phox}$, and the catalytic subunit $gp91^{phox}$. By transferring the electron from nicotinamide adenine dinucleotide phosphate (NADPH) to oxygen, the active NOX generates superoxide.[16] Apocynin, a constituent of root extracts of the medicinal herb Picrorhiza kurroa,[17] is a NOX inhibitor previously shown to downregulate effectively intracellular ROS.[18,19] However, its antioxidative effect on tenocytes remains unclear. Here we aim to evaluate the in vitro effects of apocynin on high glucose-induced oxidative stress on tenocytes.

Methods

Cell culture and experimental protocol. We conducted all experimental procedures in accordance with the approval and guidance of the Animal Care and Use Committee of our institution. We used Sprague-Dawley (S-D) rats aged eight weeks in this study.

We excised the Achilles tendons from S-D rats and soaked the excised tendons in povidone-buffered saline (PBS). We cut each tendon into small pieces sized approximately 1.5 mm^3 to 2.0 mm^3. We placed each piece individually in a well of a six-well culture plate. We propagated cells in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, Utah, USA), adding 10% fetal bovine serum (FBS; Cansera, Toronto, Canada), 100 U/ml penicillin, and 100 µg/ml streptomycin to each well. We then incubated the explants at 37°C in a humidified atmosphere of 5% CO_2/95% air. After they had attained a subconfluent state, we subcultured the cells after trypsin digestion. We changed the medium every five days. In this study, we used cells from passage 2 to 3. We seeded tenocytes onto 12-well culture plates at 10^5 cells per well and then incubated them in DMEM of two different glucose concentrations: 12 mM in the regular-glucose (RG) group, and 25 mM in the high-glucose (HG) group without FBS, to avoid overgrowth. We dissolved apocynin (Tokyo Chemical Industry, Tokyo, Japan) in dimethyl sulfoxide (DMSO) to obtain a 2 mM stock solution and diluted it into a final concentration of 100 µM. We added apocynin at cell seeding, dividing the tenocytes into four groups: the control group; RG with apocynin (RG apo+); HG with apocynin (HG apo+); and HG without apocynin (HG apo–).

Table I. Primer sequences used for polymerase chain reaction

Gene	Oligonucleotide sequence
NOX1	Forward 5' GTGGCTTTGGTTCTCATGGT 3'
	Reverse 5' TGAGGACTCCTGCAACTCCT 3'
NOX4	Forward 5'GGGCCTAGGATTGTGTTTGA 3'
	Reverse 5'CTGAGAAGTTCAGGGCGTTC 3'
IL-6	Forward 5'GGTCTTCTGGAGTTCCGTTTC 3'
	Reverse 5'GGTCTTGGTCCTTAGCCACTC 3'
GAPDH	Forward 5'GGTGGTCTCCTCTGACTTCAACA 3'
	Reverse 5'GTTGCTGTAGCCAAATTCGTTGT 3'

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-6, interleukin-6; NOX, nicotinamide adenine dinucleotide phosphate oxidase.

Quantitative real-time polymerase chain reaction analysis. At 48 hours, we extracted total RNA from tenocytes using an RNeasy Mini Kit (Qiagen, Valencia, California, USA). Using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA), we reverse-transcribed total RNA into single-strand complementary DNA (cDNA). We performed real-time polymerase chain reaction (PCR) in triplicate on the cDNA with an Applied Biosystems 7900HT Fast Real-Time PCR System and SYBR Green reagents (Applied Biosystems). We normalized results to housekeeping gene expression levels and expressed them relative to the control culture levels using the $2^{-\Delta\Delta Ct}$ method. We have listed the primer sequences in Table I.

Cell proliferation assay. We measured cell proliferation by a water-soluble tetrazolium salt (WST) assay using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Briefly, we used 96-well plates, seeding each well with 2,000 cells and filling each with 100 µl of medium. We cultured each 96-well plate in a CO_2 incubator at 37°C before the WST assay evaluation. We exposed the cells to four media (control group, RG apo+, HG apo–, HG apo+) for 48 hours. For the WST assay, we supplemented each well with 10 µl of WST for four hours at 37°C in a CO_2 incubator before spectrophotometric evaluation. We

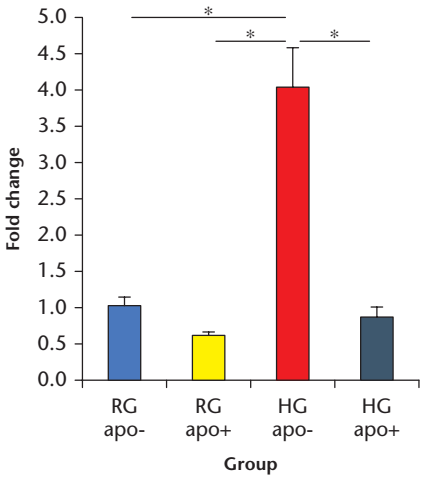

Fig. 1a

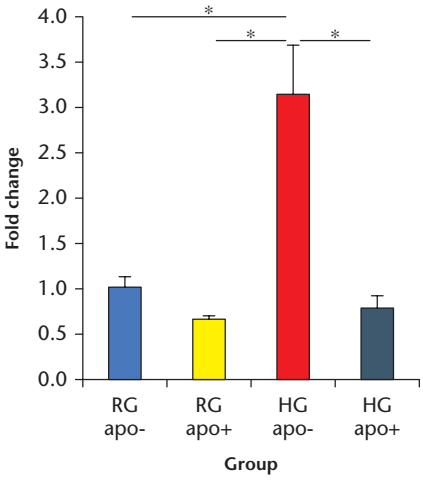

Fig. 1b

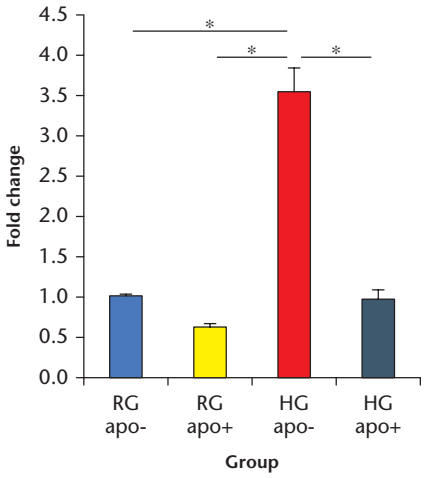

Fig. 1c

a) Expression of nicotinamide adenine dinucleotide phosphate oxidase 1 (NOX1) messenger RNA (mRNA) in the high-glucose without apocynin (HG apo–) group was significantly higher than that in the regular-glucose without apocynin (RG apo–) and regular-glucose with apocynin (RG apo+) groups at 48 hours. Its expression in the high-glucose with apocynin (HG apo+) group was significantly lower than that in the HG apo– group. However, there was no difference within the regular-glucose (RG) groups. b) Expression of NOX4 mRNA, and c) Expression of interleukin-6 (IL-6) mRNA showed similar findings to that of NOX1. *$p < 0.05$.

spectrophotometrically measured the conversion of WST to formazan at 450 nm.

Immunofluorescence staining for analysis of apoptotic cells. We detected nuclear fragmentation by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining with an APO-DIRECT Kit (Phoenix Flow Systems, San Diego, California, USA) according to the manufacturer's protocol, using fixed cells (4% paraformaldehyde/PBS) with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI). For quantitative measurement, we counted apoptosis-positive cells and DAPI-positive cells in four rectangular areas (0.75 mm × 1.0 mm) in each slide, and calculated the mean values. We calculated the percentage of apoptosis-positive cells using the formula (number of apoptosis-positive nuclei/number of DAPI-positive nuclei) × 100 and expressed it as a mean of the four areas.

Measurement of reactive oxygen species. We detected intracellular ROS levels in tenocytes by the oxidation-sensitive fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA) using the Total ROS/Superoxide Detection Kit (Enzo Life Science, Farmingdale, New York, USA) according to the manufacturer's protocol. We incubated tenocytes (5×10^4) with DCFH-DA at a final concentration of 10 µM for 60 minutes at 37°C in the dark. We washed the tenocytes three times with PBS, and then trypsinized and resuspended them. For quantitative measurement, we counted ROS-positive cells and DAPI-positive cells in four rectangular areas (0.75 mm × 1.0 mm) in each slide and calculated the mean values. We calculated the percentage of ROS-positive cells using the formula (number of ROS-positive nuclei/number of DAPI-positive nuclei) × 100 and expressed it as a mean of the four areas.

Statistical analysis. We expressed data as means (SD) and analyzed them by one-way analysis of variance. We performed post hoc analysis by Fisher's protected least significant difference (LSD) test. We considered $p < 0.05$ to indicate a statistically significant difference.

Results

Quantitative real-time PCR. At 48 hours, the expression of NOX1, NOX4, and IL-6 in the HG apo– group was significantly higher than that in the control group ($p < 0.05$, $p < 0.05$, $p < 0.05$, respectively) and in the RG apo+ group ($p < 0.05$, $p < 0.05$, $p < 0.05$, respectively) (Figure 1). The expression of these markers in the HG apo+ group was significantly suppressed compared with that in the HG apo– group ($p < 0.05$, $p < 0.05$, $p < 0.05$, respectively) (Figure 1). The control group and the RG apo+ group did not differ significantly (Figure 1).

Cell proliferation assay. At 48 hours, cell proliferation in the RG apo+ group was significantly higher than in the control group ($p < 0.05$) (Figure 2), and was significantly higher in the HG apo+ group than in the HG apo– group ($p < 0.05$) (Figure 2).

Apoptotic cell analysis. We observed apoptotic cells in both the HG apo– group and the HG apo+ group. However, we found few apoptotic cells in either the control group or the RG apo+ group (Figure 3). We observed abnormal nuclear morphology – such as nuclear fragmentation – in apoptotic cells. Figure 4 shows a quantitative analysis of the apoptotic cells. The number of apoptotic cells in the HG apo+ group was lower than in the HG apo– group,

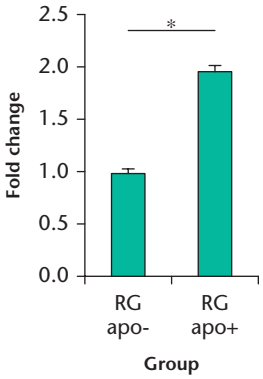

Fig. 2a

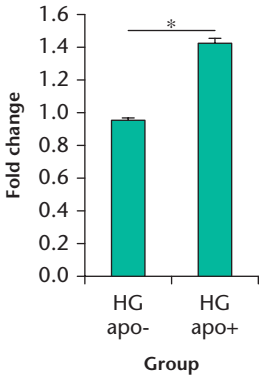

Fig. 2b

a) At 48 hours, cell proliferation in the regular-glucose with apocynin (RG apo+) group was significantly higher than in the control group (RG apo–). b) Cell proliferation in the high-glucose with apocynin (HG apo+) group was significantly higher than that in the high-glucose without apocynin (HG apo–) group. *p < 0.05.

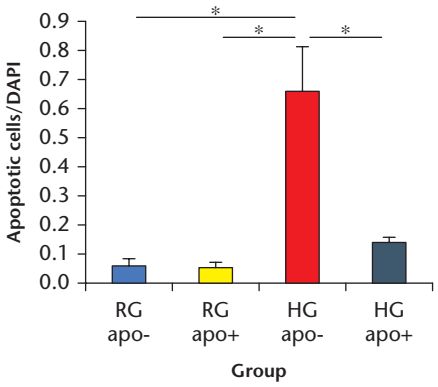

Fig. 4

Quantification of the number of apoptotic cells. The number of apoptotic cells was analyzed by fluorescence intensity normalized to cell number. The number of apoptotic cells in the high-glucose without apocynin (HG apo–) group was significantly higher than that in the regular-glucose without apocynin (RG apo–) and with apocynin (RG apo+) groups at 48 hours. The number of apoptotic cells in the HG apo+ group was significantly decreased compared with that in the HG apo– group. *p < 0.05. DAPI, 4',6-diamidino-2-phenylindole.

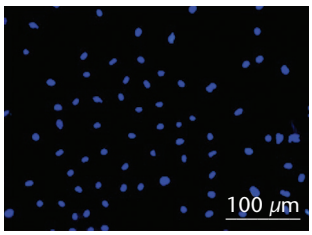

Fig. 3a

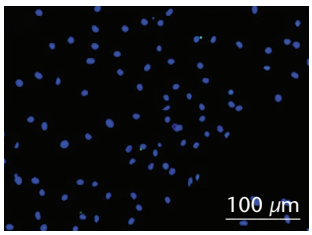

Fig. 3b

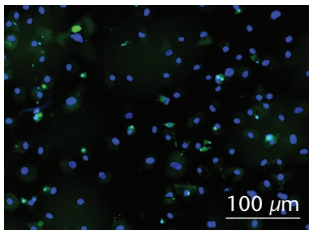

Fig. 3c

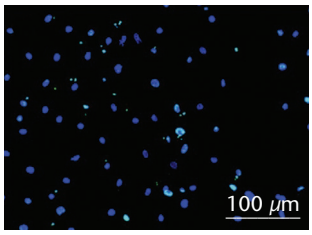

Fig. 3d

Immunofluorescence staining showing apoptotic cells (green) in each group. a) and b) There were few apoptotic cells in the regular-glucose without apocynin (RG apo–) and with apocynin (RG apo+) groups. c) and d) There was induction of apoptosis in the high-glucose (HG) groups. The number of apoptotic cells in the HG with apocynin (HG apo+) group was lower than that in the HG without apocynin (HG apo–) group.

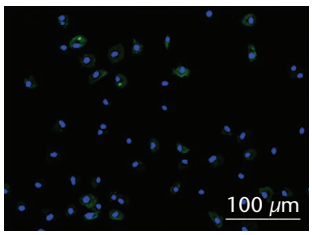

Fig. 5a

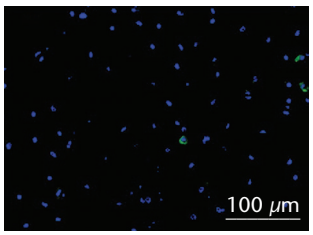

Fig. 5b

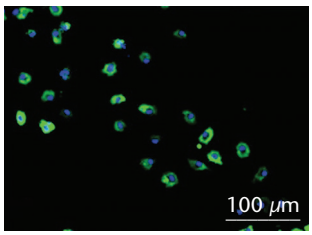

Fig. 5c

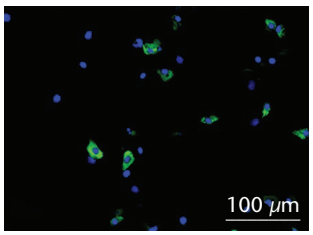

Fig. 5d

Fluorescence staining showing reactive oxygen species (ROS) accumulation (green) in tenocytes and nuclei (4' 6-diamidino-2-phenylindole) (blue). a) and b) There were few ROS accumulations in the regular-glucose without apocynin (RG apo–) and with apocynin (RG apo+) groups. c) and d) Increased ROS accumulation observed in the high-glucose (HG) groups compared with that in the regular-glucose (RG) groups. The ROS accumulation in the HG with apocynin (HG apo+) group was smaller than that in the HG without apocynin (HG apo–) group.

and there was a statistically significant difference between them. (Figure 4).

ROS-positive cells analysis. We detected intracellular ROS levels using DCFH-DA staining. The cytoplasm of ROS-positive cell was green-stained (Figure 5). We show a quantitative analysis of ROS-positive cells in Figure 4.

Reactive oxygen species accumulation of the HG apo+ group was lower than that of the HG apo– group, and there was a statistically significant difference between them (Figure 6).

In this respect, there was not a statistically significant difference between the control group and the RG apo+ group (Figure 6).

Discussion

Reports have described several pathological mechanisms regarding tendon lesion with diabetes.[1-4] In tenocytes, a high-glucose concentration upregulates the expression of matrix metalloproteinases (MMPs), which are a family of extracellular matrix (ECM)-degrading enzymes.[20] The potential combination of increased local matrix degradation brought about by enhanced MMP expression and decreased ECM production by tenocytes

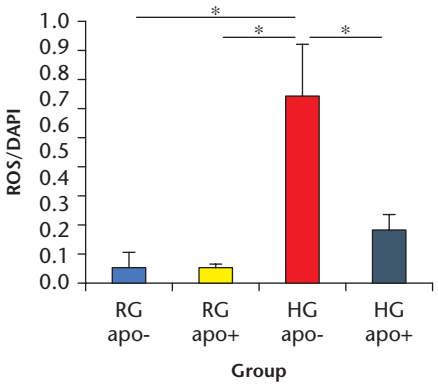

Fig. 6

Quantification of the accumulation of reactive oxygen species (ROS). ROS accumulation was analyzed by fluorescence intensity normalized to cell number. The ROS accumulation in the high-glucose without apocynin (HG apo–) group was greater than that in the regular-glucose without apocynin (RG apo–) and with apocynin (RG apo+) groups at 48 hours. Alternatively, the ROS accumulation in the high-glucose with apocynin (HG apo+) group was greater than that in the regular-glucose groups. However, there was no significant difference. The ROS accumulation in the HG apo+ group was significantly smaller than that in the HG apo– group. *$p < 0.05$. DAPI, 4′,6-diamidino-2-phenylindole.

might predispose patients with diabetes to tendinopathy or tendon injuries.[20]

In diabetic patients with flexor tenosynovitis, a small number of inflammatory cells were found in the hyperplastic granulation tissue.[21] Immunohistochemistry analysis identified both the higher density of type 1 collagen and an increase in the expression of vascular endothelial growth factor (VEGF) and increased immunostaining for nuclear factor kappa B (NFκB) p50 nuclear localization in the nucleus in the Achilles tendons of the diabetic group when compared with the control group.[22] Kameyama et al[21] reported that in diabetic patients with stenosing flexor tenosynovitis, the increase in fibrocartilage metaplasia and granulation tissue hyperplasia was more frequent than in subjects without diabetes.[21,22] Evans and Trail[23] compared human flexor tendon cells with extensor tendon cells in vitro. The study demonstrated that the cells obtained from two different types of tendon behave similarly when exposed to identical environmental conditions in vitro.[23]

Ueda et al[9] reported the upregulation of the expression of messenger RNA (mRNA) for NOX1 and interleukin-6 (IL-6) and the production of ROS by high-glucose conditions. A higher accumulation of NOX – indicating chronic inflammatory status – was also found in diabetic rat tendons.[19] High glucose levels have also been shown to stimulate ROS production through protein kinase C-dependent activation of NOX in cultured aortic smooth muscle cells and endothelial cells.[24]

In the present study, expression of NOX1, NOX4, and IL-6 was higher in high-glucose conditions, which supports the findings of previous reports.[9] Apocynin has previously been shown to effectively inhibit increased NOX activity in diabetic aortas and restore changes in nitric oxide synthase expression, thereby blocking the cycle resulting in diabetes-associated endothelial dysfunction.[25] Li et al[26] reported that in the testes of diabetic rats, apocynin reduced the production of ROS and apoptotic cells, and there were statistically significant differences.

The present study using rat tenocytes also showed the administration of apocynin to reduce the expressions of NOX1, NOX4, and IL-6 in high-glucose conditions. Quantitative analysis of ROS production also showed that treatment with apocynin reduced ROS production under high-glucose conditions.

The effects of hyperglycaemia on cell proliferation differ based on the cell types.[27-30] Higuchi et al[28] reported that glucose stimulates cell proliferation of rat peritoneal fibroblasts. There is a growing body of evidence showing that excessive ROS production triggers cellular damage and an apoptosis cascade through the phosphorylation of c-Jun N-terminal kinases (JNKs) and activation of Bax,[11,31] and by the activation of caspase and the regulation of the expression of B-cell lymphoma 2 (Bcl-2) family proteins.[32] The overproduction of ROS promotes the process of apoptosis by both increasing caspase-3 activity and inhibiting Bcl-2 expression, clearly demonstrating the crosstalk that exists between oxidative stress and apoptosis.[33,34]

Poulsen et al[35] reported that in human tenocytes, oxidative stress by hydrogen peroxide has been demonstrated to induce apoptosis via Bim, which is proapoptotic protein, –mediated apoptosis through miR-28-5p and p53 upregulation in high-glucose conditions. Therefore, hyperglycaemia might inhibit the ability to repair the damaged or degenerated tendon.

In the present study, in high-glucose conditions, apocynin ameliorated tenocyte viability, and there was a statistically significant difference, due to its antioxidant and anti-inflammatory effects.

There are several limitations to this study. First, the results of this study were preliminary, and clinical application in humans would require further detailed research. Researchers should be cautious when extrapolating the findings of this in vitro study to in vivo conditions, and further animal studies should be conducted to validate the effects of apocynin on oxidative stress in diabetic tenocytes. There are several pathways regarding apoptosis under high-glucose conditions, and the mediator of the apoptotic signalling pathways was not investigated. We used tenocytes cultured at passage 2 or 3; however, phenotypic drift should be taken into consideration because the phenotype of tenocytes in culture rapidly drifts with progressive passage.[36] An experiment using an apocynin inhibitor might be needed for further understanding of the mechanism.

In conclusion, high-glucose concentrations upregulated the mRNA of NOX1, NOX4, and IL-6 expression and the production of ROS. High-glucose concentrations also increased the number of apoptotic cells. Apocynin reduced ROS production and cell death via NOX

inhibition and increased cell viability in high-glucose conditions. Apocynin is thus a potential prodrug in the treatment of diabetic tendinopathy.

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