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Hyperglycaemia Attenuated C2C12 Myoblast Proliferation and Induced Skeletal Muscle Atrophy via Modulating Myogenic Regulatory Factors Genes Expression in Diabetic Rats

(Hiperglisemia Dilemahkan C2C12 Pembiakan Mioblas dan Atrofi Otot Rangka Terinduksi melalui Modulasi Faktor Kawalaturan Miogenesis Ekspresi Gen dalam Tikus Diabetis)

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ABSTRACT

Diabetes mellitus is characterised by high blood glucose level termed hyperglycaemia (HG). It has been reported to affect skeletal muscle by inducing skeletal muscle atrophy and skeletal muscle protein degradation leading to impairment of muscle function. This study aimed to investigate the effects of HG on the expression of myogenic regulatory factor genes in muscle progenitor cells and in skeletal muscle. The number of C2C12 myoblasts cultured in HG condition was significantly decreased compared to control in dose and time dependent manner. In addition, the number of Ki-67 positive nuclei was significantly decreased after treatment under HG condition. Real time PCR showed significant suppression of MyoD and myogenin, while Myf5 gene expression was significantly enhanced, compared to control. Furthermore, histological examinations of muscle fibres showed atrophy of tibialis anterior (TA) muscle from diabetic rats. The frequency of distribution of muscle fibre cross-sectional area (MCA) in diabetic rats was shifted leftward from that of normal control rats. In contrast, the MyoD and myogenin expression in TA muscle of diabetic rats were significantly increased compared to normal control rats. This study provides novel knowledge on the changing myogenic regulatory factor gene expression in hyperglycaemic condition, both *in vitro* and *in vivo*, leading to skeletal muscle atrophy.

Keywords: Atrophy; diabetes; myoblast; myogenesis; skeletal muscle

ABSTRAK

Diabetes mellitus dicirikan oleh tahap glukosa darah yang tinggi dipanggil hiperglisemia (HG). Ia telah dilaporkan menjejaskan otot rangka dengan mengaruh atrofi otot rangka dan degradasi protein otot rangka yang membawa kepada kemerosotan fungsi otot. Kajian ini bertujuan untuk mengkaji kesan HG ke atas pengekspresan gen faktor pengawalaturan miogenik dalam sel progenitor otot dan dalam otot rangka. Bilangan mioblas C2C12 yang dibiakkan dalam keadaan HG berkurangan dengan ketara berbanding dengan kawalan yang bergantung kepada dos dan masa. Di samping itu, bilangan nukleus positif Ki-67 berkurangan dengan ketara selepas rawatan dalam keadaan HG. PCR masa nyata menunjukkan penindasan signifikan MyoD dan miogenin, manakala pengekspresan gen Myf5 meningkat dengan signifikan, berbanding kawalan. Tambahan pula, pemeriksaan histologi gentian otot mendedahkan atrofi otot tibialis anterior (TA) bagi tikus diabetes. Kekerapan taburan kawasan keratan rentas gentian otot (MCA) pada tikus diabetes dianjak ke kiri berbanding tikus kawalan normal. Sebaliknya, pengekspresan MyoD dan miogenin dalam otot TA tikus diabetes meningkat dengan ketara berbanding tikus kawalan normal. Kajian ini memberikan pengetahuan baharu tentang perubahan pengekspresan gen faktor pengawalaturan miogenik dalam keadaan hiperglisemik yang membawa kepada atrofi otot rangka bagi kedua-dua keadaan *in vitro* dan *in vivo*.

Kata kunci: Atrofi; diabetes; mioblas; miogenesis; otot rangka

Introduction

Skeletal muscle consists of myofibre arranged side by side and has major roles in body movement and metabolism. The force of muscle contraction mainly depends on the size of myofibres (Krivickas et al. 2011). It has been reported that diabetes mellitus affects skeletal muscle by inducing skeletal muscle atrophy (Hirata et al. 2019; Surinlert et al. 2021) and skeletal muscle protein degradation (Robinson et al. 2016) leading to impairment of muscle function. It has been proposed that hyperglycaemia promotes skeletal muscle atrophy via a WWP1/KLF15 pathway (Hirata et al. 2019). However, the precise mechanism of the effect of diabetes on skeletal muscle architecture is still unclear.

Myogenic regulatory factors (MRF) genes are the master regulatory factors that are constitutively expressed in skeletal muscle since early stage until maturation of muscle development (Asfour, Allouh & Said 2018). These include MyoD, myogenin, Myf5 and MRF4, which confer myogenesis. In the early stage of development, the progenitor cells increase the expression of Myf5, MyoD and MRF4, and become myoblasts. These myoblasts further differentiate into myocytes and finally fuse together to form multinucleated muscle fibres under the control of myogenin, MyoD and MRF4 genes (Shirakawa et al. 2022). In mature skeletal muscle, these MRF genes play important roles to influence myosin heavy chain content and metabolic properties of myofibres and suppress hypertrophy of skeletal muscle (Zammit et al. 2017).

The expression of MRF genes has been reported to be influenced by several stimuli, including chemical toxicants (Culbreth & Rand 2020), ageing (Di Filippo et al. 2016; Sosa et al. 2021), physical activity (Caldow et al. 2015) and muscular diseases (Spassov et al. 2011). Reactive oxygen species molecules have also been reported to be the important mediators that disrupt the normal expression of MRF genes in many conditions (Acharya et al. 2013; Di Filippo et al. 2016).

Since hyperglycaemic condition has been reported to increase reactive oxygen species leading to cellular oxidative stress (Matsumoto et al. 2021), this study, therefore, aimed to investigate the effects of hyperglycaemia (HG) on the expression of myogenic regulatory factor genes in muscle progenitor cells and in skeletal muscle.

Materials and Methods

Reagents

Unless otherwise indicated, cell culture media were from Life Technologies (Grand Island, NY, USA). Other basic chemicals were from Sigma (St. Louis, MO, USA). Anti-Ki-67 antibody from Abcam (Cambridge, MA, USA) was used in this experiment.

Cell culture and treatments

C2C12 mouse myoblast cell line (ATCC; Manassas, VA, USA) were cultured in DMEM, supplemented with 10% foetal bovine serum (FBS) in a humidified CO_2 incubator at 37 °C. To test the effect of hyperglycaemia on myoblast proliferation, C2C12 myoblasts were seeded into 6 wells-plates and were allowed to grow for 24 h. The growing cells were shifted to growth medium containing 2-deoxy ribose at 0, 10, 15, 20 and 25 mM for 24 to 72 h. The treated cells at 48 h were then trypsinised for cell counting or were fixed with ice-cold methanol for immunostaining. The cells cultured in growth medium (control) and growth medium containing 25 mM 2-deoxyribose for 48 h were analysed using real-time PCR.

Animals and treatments

Male Wistar rats (8 weeks old) were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University (Hat-Yai, Thailand). The animals were housed in a 12-h light/dark cycle at 25±2 °C, with food and water *ad libitum.* To induce hyperglycaemic condition, the rats were intraperitoneally injected with streptozotocin (60 mg/kg) dissolved in citrate buffer. After 72 h post injection, blood samples from the tail vein were collected and were measured for blood glucose levels using blood glucose meter (Roche diagnostic, Mannheim, Germany). The rats with blood sugar level higher than 2.5 g/L were allocated as hyperglycaemic group (HG). The rats in control group (Ctrl) were injected with the citrate buffer. These rats were cared for 8 weeks before being euthanised and the tibialis anterior (TA) muscles were removed for histological examinations. The animal protocol in this study was specifically reviewed and approved by the Animal Ethical Committee of Prince of Songkhla University (Songkhla, Thailand) (approval ID: 2562-01-006).

Cell counting

The treated C2C12 cells were trypsinised and resuspended in 1 mL medium. The resuspended cells were mixed with trypan blue dye at 1:9 ratio. The mixture was gently mixed by pipetting and left to stand at room temperature for 5 min. The mixture (20 μL) was then applied into a haemocytometer counting chamber and were counted under a light microscope. The counting cells were calculated and expressed as total cell number.

IMMUNOFLUORESCENCE STAINING

The treated C2C12 cells were fixed with ice-cold methanol for 10 min. The fixed cells were washed and rehydrated in phosphate buffered saline (PBS). The cells were permeabilised and non-specific binding was blocked with 5% goat serum in 0.3% TritonX-100 diluted in PBS for 1 h at room temperature (RT) with constant agitation. The cells were subjected to incubation with anti-Ki-67 antibody overnight at 4 °C. Excess antibody was washed with PBS and the cells were incubated with Alexa488-conjugated secondary antibody and Hoechst 33342 diluted in PBS for 1 h at RT. After several washes, the signal was visualised under a fluorescence microscope (Olympus IX73, Tokyo, Japan). The number of Ki-67 positive nuclei were counted and expressed as percentage of total nuclei.

HISTOLOGICAL STUDY

The TA muscles were dissected and immediately fixed in 10% formalin overnight. Tissues were embedded in paraffin and sectioned at 5 µm. The sections were stained with haematoxylin and eosin (H&E). The histology of skeletal muscle was observed under a light microscope and muscle fibre cross-sectional area (MCA) was measured with CellSens Dimension software.

REAL-TIME POLYMERASE CHAIN REACTION (REAL-TIME PCR)

The treated C2C12 myoblasts and TA muscles were subjected to RNA extraction and were then purified using RNeasy MiniKit (Qiagen). The cDNAs were synthesised from 2.5 ug of each total RNA sample with SuperScript III reverse transcriptase (Invitrogen) using oligo(dT) primers. Real-time PCR was performed using Luna universal RT-qPCR on a BioRad CFX96 Touch Real Time PCR machine. The primer sequences were designed for MyoD (Fw 5-ATGATGACCCGTGTTTCGACT-3' and Rw 5-CACCGCAGTAGGGAAGTGT-3'), Myf-5 (Fw 5'-GCCTTCGGAGCACACAAAG-3' and Rw 5'-TGACCTTCTTCAGGCGTCTAC-3'), myogenin (Fw 5'-GAGACATCCCCCTATTTCTACCA-3' and Rw 5'-GCTCAGTCCGCTCATAGCC-3'), MRF4 (Fw 5'-CTGAAGCGTCGGACTGTGG-3' and Rw 5'-ATCCGCACCCTCAAGAATTTC-3'), and GAPDH (Fw 5'-TGCGACTTCAACAGCAACTC-3' and Rw 5'-GCCTCTCTTGCTCAGTGTCC-3') (Quinn et al. 2017; Shahini et al. 2018). The gene expressions were normalised to GAPDH and expressed using $2^{-\Delta\Delta CT}$ method.

STATISTICAL ANALYSIS

The results were expressed as means ± SEM from at least three independent experiments. Statistical differences were analysed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The Student's T-test was also used in this experiment, when appropriate. The statistical analysis was performed with GraphPad Prism version 5.00. Statistical significance was displayed as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

HYPERGLYCAEMIA INHIBITED C2C12 MYOBLAST CELL PROLIFERATION

Cell counting showed a significant decrease in cell number after treatment with 20 to 30 mM 2-deoxy ribose for 24 h. After prolonged treatment for 48 to 72 h, the 10 to 15 mM groups showed significant differences, the degree of which was further increased in the 20 to 30 mM groups, compared to control (Figure 1(A)). In addition, the percentages of Ki-67 positive nuclei in the 15 to 30 mM groups after 48 h treatment were significantly decreased, compared to the non-treated control (Figure 1(B) and 1(C)). These results indicated that the myoblast proliferation was inhibited under hyperglycaemic condition in dose and time dependent manners.

HYPERGLYCAEMIA INHIBITED MYOBLAST PROLIFERATION BY MODULATING MRFS GENES EXPRESSION

The results showed that hyperglycaemic condition significantly suppressed the expressions of MyoD and myogenin but significantly upregulated Myf5 gene expression up to 2.5 folds. On the other hand, there was no effect of hyperglycaemia on MRF4 expression in myoblasts (Figure 2).

HYPERGLYCAEMIA INDUCED SKELETAL MUSCLE ATROPHY

To evaluate the effect of hyperglycaemia *in vivo*, the H&E staining of TA muscles was performed. The results showed marked atrophy of the skeletal muscle fibre in the diabetic group (Figure 3(A)). The muscle fibre cross-sectional areas (MCA) were also measured and reported as frequency distribution of MCA. There was a leftward shift in the distribution of MCA in TA muscle from the diabetic group compared to the non-diabetic control (Figure 3(B)).

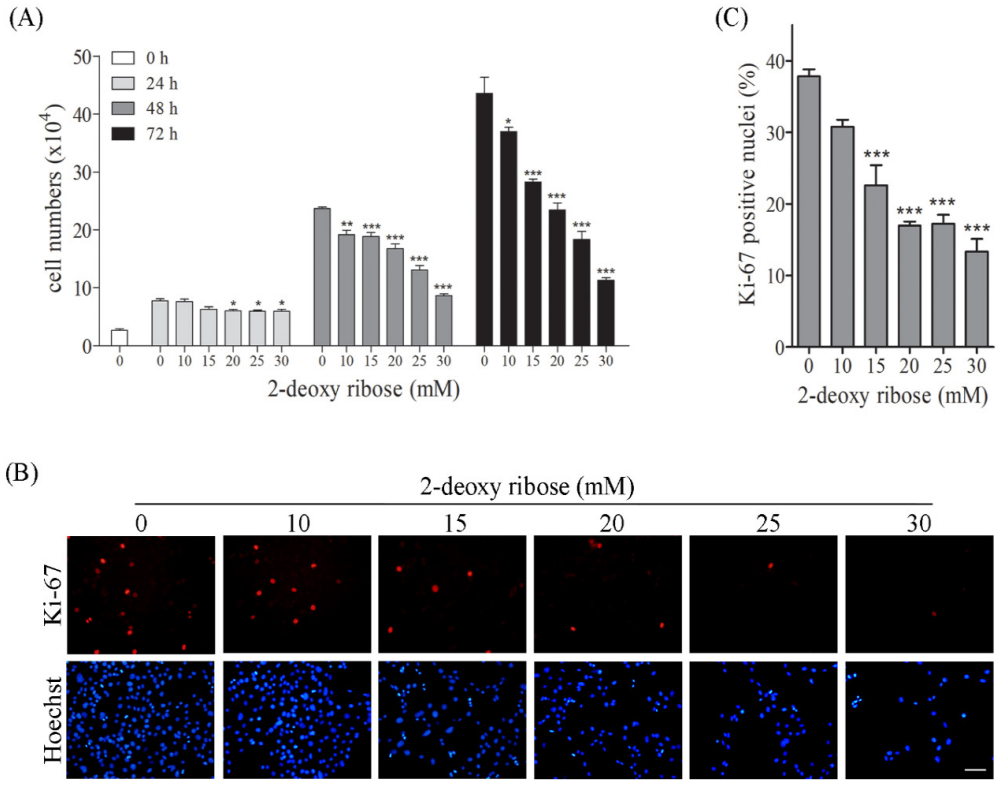

FIGURE 1. The subconfluence C2C12 myoblasts were cultured in growth media containing 2-deoxy ribose at the indicated concentrations for 24 to 72 h. After the treatments, cell numbers were counted using haemocytometer (A) and were fixed for anti-Ki-67 staining at 48 h (B). The number of Ki-67 positive nuclei (red) were reported as percentage of total nuclei (blue) stained with Hoechst 33342 (C). Scale bar = 50 µm, *P < 0.05, **P < 0.01, ***P < 0.001 compared to the control

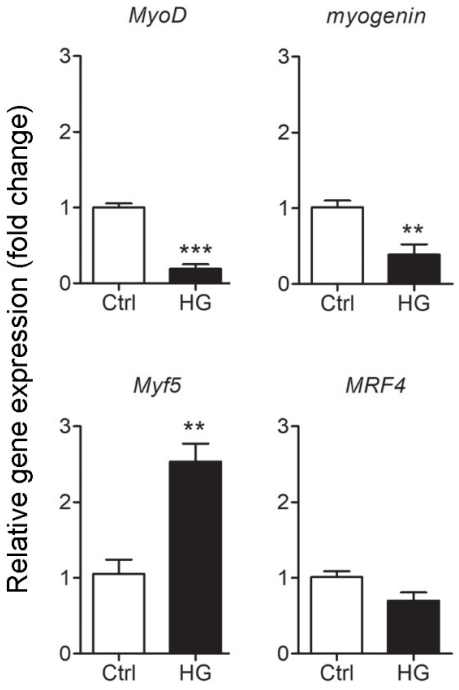

FIGURE 2. The expression of myogenic regulatory factor genes (MyoD, myogenin, Myf5, MRF4) for C2C12 myoblasts cultured in growth medium (control; ctrl) or growth medium containing 25 mM 2-deoxyribose (hyperglycaemia, HG) for 48 h were analysed by real-time PCR. **P < 0.01, ***P < 0.001 compared to the control

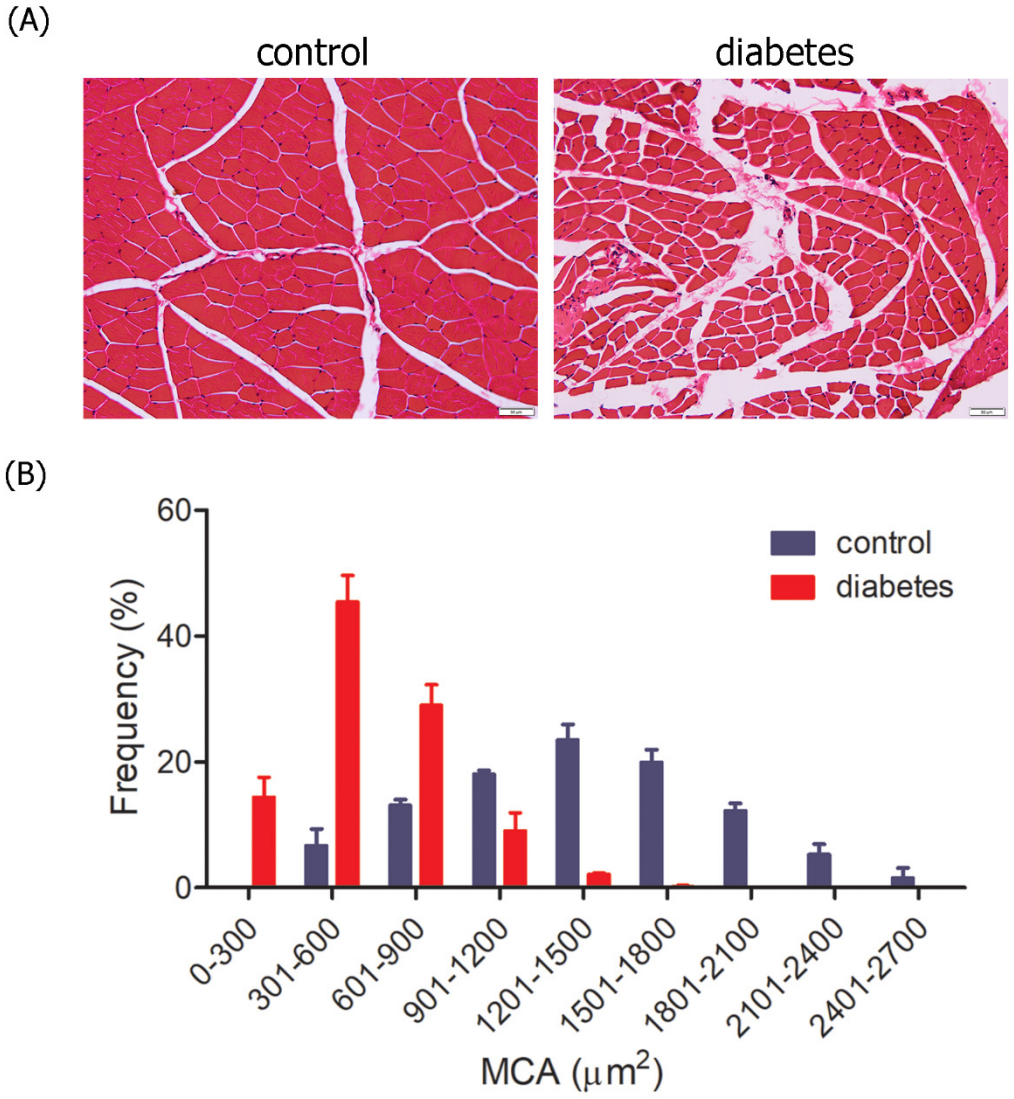

FIGURE 3. The tibialis anterior muscle from diabetic and non-diabetic control (8-week) were processed for H&E staining. Representative photographs of tibialis anterior muscle sections (A) and myofibre size distribution (B)

HYPERGLYCAEMIA INDUCED MUSCLE ATROPHY BY MODULATING MRF GENE EXPRESSION

Real-time PCR analyses of MRF genes from TA muscle of diabetic group showed significant changes compared to those of the control group. The expression levels of MyoD and myogenin in TA muscles of the diabetic group were significantly upregulated up to 1.5 and 2.5 folds, respectively. Surprisingly, the expression of Myf5 and MRF4 in TA muscle of diabetic group was not significantly altered compared to control group (Figure 4).

DISCUSSION

In this study, hyperglycaemia strongly inhibited myoblast cell proliferation, resulting in decreased cell numbers and a decrease in the expression of the proliferation marker Ki-67. It has been reported that satellite cells have the unique feature that their proliferation was impeded by high glucose concentrations (Furuichi et al. 2021). Our results were in agreement with the previous reports that hyperglycaemia inhibited proliferation in endothelial cells (Qiu et al. 2020),

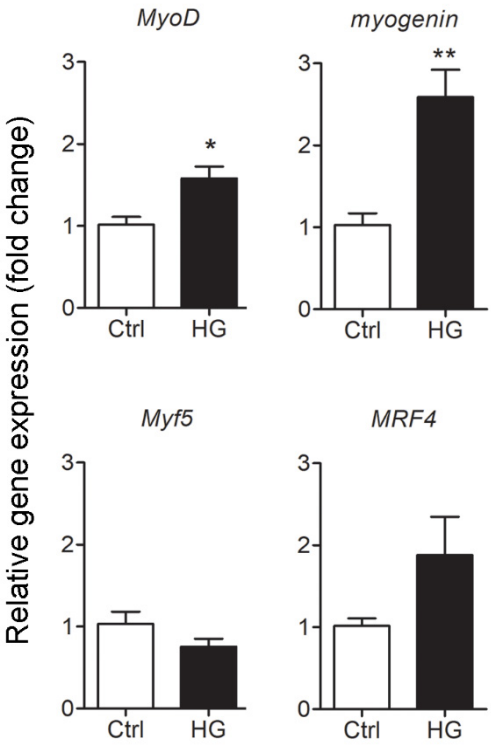

FIGURE 4. The expression of the myogenic regulatory factor genes (MyoD, Myf5, myogenin, MRF4) in tibialis anterior muscle of control and diabetic groups (8-week) were analysed by real-time PCR. *P < 0.05, **P < 0.01 compared to the control

human gingival fibroblasts (Buranasin et al. 2018) and human dental pulp cells (Horsophonphong et al. 2020). However, several cell types were increased in cell proliferation when cultured in high glucose condition, including human pancreatic duct epithelial cells (Ito et al. 2017), endometrial cancer cells (Han et al. 2015) and breast cancer cells (Hou et al. 2017). This discrepancy may depend on the cell type being tested. It has been shown that hyperglycaemic condition down-regulated the expression of PI3k and Akt signalling and finally led to proliferative dysfunction in human umbilical vein endothelial cells (Varma et al. 2005), likely due to increased intracellular ROS production in the human gingival fibroblast cells (Buranasin et al. 2018). The activation of PI3K/Akt-kinase pathway is a critical regulator of cell proliferation by regulating cyclin D1, cyclin D2 and p21 levels and cyclin-dependent minase-4 activity (Fatrai et al. 2006). Inactivation of PI3K/AKT would abolish the cascade of cell proliferation. Other possible mechanisms of hyperglycaemia to inhibit cell proliferation may occur via the accumulation of intracellular free radicals, which in turn lead to cellular oxidative stress (Schieber & Chandel 2014). The oxidative stress has been reported to induce DNA damage and induce cell cycle arrest by suppression of cyclin D and CDK4 expression, which in turn disrupt cell proliferation (Pizarro et al. 2009). Moreover, cellular stress has been reported to inhibit myoblast cell proliferation by inducing cell cycle arrest at S-phase, finally leading to apoptosis (Surinlert et al. 2020).

The downregulation of MyoD and myogenin expression in myoblasts cultured in hyperglycaemic condition have not been reported before. Decreases in the expression of mRNA levels of MyoD and myogenin indicated that hyperglycaemia suppressed the myogenesis of C2C12 cells. These changes may be the adaptive response of myoblasts to compromise with the apoptosis induction because MyoD downregulation has been reported to increase cellular resistance to apoptosis (Asakura et al. 2007; Hirai et al. 2010) by down-regulation of microRNA-1 (miR-1) and miR-206 and by upregulation of Pax3 (Hirai et al. 2010). These

microRNAs have been reported to be important regulators of biological processes, including metabolism, cell growth and apoptosis (Poy, Spranger & Stoffel 2007; Wüst et al. 2018; Zheng et al. 2018) while Pax3 has major roles in muscle formation, specification, homeostasis and repair (Relaix et al. 2021). On the other hand, myogenin has been reported to be an essential regulator for the muscle progenitor cell homeostasis (Ganassi et al. 2020). The down-regulation of myogenin would therefore interfere with normal cellular activities, including cell proliferation. In contrast, Myf5 expression was upregulated. The elevation of Myf5 expression may be a compensation mechanism to maintain the proliferative status as Myf5 functions toward myoblast proliferation (Ishibashi et al. 2005). The Myf5 promoted the myoblast proliferation by enhancing Cyclin D1 mRNA translation (Panda et al. 2016).

For *in vivo* study, rat model mimicking type I diabetes with impaired insulin secretion was generated. The results showed that hyperglycaemia induced skeletal muscle atrophy. These results were consistent with previous research where hyperglycaemia induced atrophy in tibialis anterior muscle (Cai et al. 2021; Surinlert et al. 2021), gastrocnemius and extensor digitorum longus muscles (Hirata et al. 2019). The possible mechanism has been proposed to occur through inhibition of the expression of WW domain-containing E3 ubiquitin protein ligase 1 (WWP1), which in turn decelerates degradation of Krüppel-like factor 15 (KLF15) molecules. KLF15 finally binds to target genes mediating protein catabolism, decreased muscle mass and mean muscle fibre area (Hirata et al. 2019) leading to the leftward shift distribution of muscle fibre cross-sectional area in hyperglycaemic rats (Cai et al. 2021). In addition, hyperglycaemia has been reported to cause oxidative stress in skeletal muscle (Bravard et al. 2011), which was implicated with skeletal muscle atrophy (Huang et al. 2019), decreased skeletal muscle mass and strength (Zacarías-Flores et al. 2018), suppressed protein synthesis and promoted muscle protein breakdown (Powers, Smuder & Criswell 2011).

In contrast to myoblasts, the expression of MyoD and myogenin in TA muscle of diabetic rats was upregulated. The upregulation may be the response to maintain muscle mass and functions. Since MyoD and myogenin have been reported to be important in regulating muscle mass (Aguiar et al. 2013), essential for adult myofibre growth (Ganassi et al. 2020) and the regulation of muscle oxidative metabolism (Shintaku et al. 2016).

Of note, the increases in the expressions of MyoD and myogenin in diabetic skeletal muscle were similar to the skeletal muscle of ageing and senile rats which were thought to be due to insufficient motor innervation (Always & Lowe 2001; Dedkov et al. 2003). Indeed, myogenin has been reported to be upregulated in atrophic muscles caused by denervation (Macpherson, Wang & Goldman 2011). This notion was in agreement with the previous reports that hyperglycaemia induced sensorimotor dysfunction and axon degeneration (Muller et al. 2008), axon dysfunction (Arnold, Kwai & Krishnan 2013) and impairment of axonal transport (Baptista et al. 2019). Thus, peripheral neuropathy caused by diabetes leads to skeletal muscle function decline (Parasoglou, Rao & Slade 2017).

Conclusions

This study analysed the effect of hyperglycaemia on myoblast cells and skeletal muscle. Hyperglycaemia was found to attenuate myoblast cell proliferation by suppressing MyoD and myogenin gene expression. However, hyperglycaemia induced skeletal muscle atrophy by enhancing MyoD and myogenin gene expression. This study contributed new information on the changes in the expression of myogenic regulatory factor genes under both *in vitro* and *in vivo* hyperglycaemic condition which result in skeletal muscle atrophy. This information is useful for further development of drugs to prevent muscle atrophy in diabetes patients. Nonetheless, further studies are required to identify the mechanism of hyperglycaemia in modulating myogenic regulatory factor gene expression.

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