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ORIGINAL PAPER

High glucose-induced oxidative stress promotes autophagy through mitochondrial damage in rat notochordal cells

Eun-Young Park · Jong-Beom Park

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Abstract
Purpose Diabetes mellitus is associated with an increased risk of intervertebral disc degeneration (IDD). Reactive oxygen species (ROS), oxidative stressors, play a key role in autophagy of diabetes-associated diseases. Mitochondria are known to be the main source of endogenous ROS in most mammalian cell types. The authors therefore conducted the following study to evaluate the effects of high glucose concentrations on the induction of oxidative stress and autophagy through mitochondrial damage in rat notochordal cells.
Methods Rat notochordal cells were isolated, cultured, and placed in either 10 % fetal bovine serum (normal control) or 10 % fetal bovine serum plus two different high glucose concentrations (0.1 M and 0.2 M) (experimental conditions) for one and three days, respectively. We identified and quantified the mitochondrial damage (mitochondrial transmembrane potential) and the generation of ROS and antioxidants (manganese superoxide dismutase [MnSOD] and catalase). We also investigated expressions and activities of autophagy markers (beclin-1, light chain3-I [LC3-I] and LC3-II, autophagy-related gene [Atg] 3, 5, 7, and 12).
Results An enhanced disruption of mitochondrial transmembrane potential, which indicates mitochondrial damage, was identified in rat notochordal cells treated with both high glucose concentrations. Both high glucose concentrations increased production of ROS by rat notochordal cells in a dose- and time-dependent manner. The two high glucose solutions also enhanced rat notochordal cells' compensatory expressions of MnSOD and catalase in a dose- and time-dependent manner. The proautophagic effects of high glucose concentrations were manifested in the form of enhanced rat notochordal cells' expressions of beclin-1, LC3-II, Atg3, 5, 7, and 12 in a dose- and time-dependent manner. The ratio of LC3-II/LC3-I expression was also increased in a dose- and time-dependent manner.
Conclusions The findings from this study demonstrate that high glucose-induced oxidative stress promotes autophagy through mitochondrial damage of rat notochordal cells in a dose- and time-dependent manner. These results suggest that preventing the generation of oxidative stress might be a novel therapeutic target by which to prevent or to delay IDD in patients with diabetes mellitus.

Keywords Autophagy · Mitochondral damage · Diabetes · High glucose concentration · Oxidative stress · Intervertebral disc degeneration · Reactive oxygen species

Introduction

Autophagy is a physiologic mechanism in which cells degrade unnecessary or dysfunctional cellular organelles through the lysosomal machinery [1]. Proper regulation of autophagy guarantees the fine balance of synthesis, degradation and recycling of cellular components. These functions render autophagy essential for proper cell differentiation and organ development. Autophagy consists of a multi-step process that is highly regulated by autophagy-related genes (Atgs) and light chain protein 3 (LC3) [2–4]. Until now, more than 31 Atgs have been identified that encode proteins that are evolutionarily conserved and essential for the execution of autophagy. During autophagy, cytosolic microtubule-associated protein LC3-I is lipidated and converted to LC3-II, which is translocated to the autophagosomal membrane. Thus, conversion of LC3-I to LC3-II and accumulation of LC3 are widely used as markers of autophagy. Beclin-1 is a BH3 member of the Bcl-2 gene family that can drive the autophagic process in mammalian cells [5, 6]. Though autophagy is generally viewed as a survival mechanism, excessive or dysregulated autophagy has recently been implicated in the pathogenesis of

E.-Y. Park · J.-B. Park (✉)
Orthopaedic Surgery, Uijongbu St. Mary's Hospital, The Catholic University of Korea School of Medicine, Uijongbu, Korea
e-mail: spinepjb@catholic.ac.kr

various diseases including cancer, neurodegenerative and cardiovascular diseases [4, 7–10].

Hyperglycemia is a major causative factor in the development of diabetes-associated diseases. Several biochemical pathways have been associated with hyperglycemia, such as glucose-mediated increases in reactive oxygen species (ROS) [11]. It is known that mitochondria are the main source of endogenous ROS in most mammalian cell types [12]. ROS are thought to play a role in varied cellular processes, such as autophagy [13, 14]. They appear to cause cellular damage and lack a physiological function. Therefore, accumulations of ROS and oxidative stress have been associated with a variety of diseases including neurodegenerative diseases, diabetes, cancer, premature aging, and inflammatory disorders [15–17]. Diabetes mellitus is known to be an important etiological factor of intervertebral disc degeneration (IDD) [18, 19]. However, to the best of the authors' knowledge, the biological effect of diabetes mellitus on oxidative stresses, autophagy, and IDD has not been elucidated. Therefore the present study was performed to evaluate the effects of high glucose concentrations on the induction of oxidative stress and autophagy through mitochondrial damage in rat nucleus pulposus cells.

Materials and methods

Notochordal cell culture and treatment with two different high glucose concentrations

All lumbar intervertebral discs (L1-6) were harvested from four-week-old male Sprague Dawley rats (Orient Bio., Seoul, Korea) immediately after they were sacrificed. The discs were carefully dissected under a microscope to obtain only the gelatinous notochordal nucleus pulposus (NP) tissue, and the harvested NP tissue was pooled in [alpha]-minimum essential medium ([alpha]-MEM; Gibco BRL, Grand Island, NY, USA). The cells were released from the NP tissue in HBSS (Hyclone, Ottawa, Ontario, Canada) with 0.02 % pronase (Sigma-Aldrich, St. Louis, MO, USA) by vigorous pipetting. The cells were cultured in a complete medium [alpha]-MEM supplemented with 10 % fetal bovine serum (Hyclone), and 1 % penicillin-streptomycin (Gibco BRL) at 37 °C in a humidified atmosphere (95 % air, −5 % CO_2). After the cells grew to confluence, they were split once (passage 1) and grew to confluence again. The cells were then trypsinized, washed, and plated on six or 12-well culture plates. When the cells reached 80 to 90 % confluence, the notochordal cells were placed in either fetal bovine serum (FBS) (normal control) of 10 % FBS plus two different high glucose concentrations (0.1 M and 0.2 M) (experimental conditions) for one and three days. This study was approved by the Institution's Animal Care and Use Committee.

Detection of mitochondrial damage

Mitochondrial damage of the notochordal cells was detected by mitochondrial transmembrane potential apoptosis detection kit (Abcam Plc, Cambridge, UK). The kit utilizes MitoCapture™, a cationic dye that fluoresces differentially in healthy and damaged apoptotic cells. In damaged apoptotic cells, MitoCapture™ cannot aggregate in the mitochondria due to the altered mitochondrial transmembrane potential, and thus it remains in the cytoplasm in its monomer form, fluorescent green. The cells were seeded on a glass cover slip in 12-well culture plates and then incubated with the Mito-Capture™ reagent in a pre-warmed incubation buffer for 30 minutes at 37 °C in a humidified atmosphere. Fluorescent signals were detected by fluorescence microscope (Image Pro Plus) (Olympus).

Intracellular ROS measurement

Intracellular accumulation of ROS was measured by ROS detection with H_2DCF-DA (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Notochordal cells were grown in 60 mm culture dishes. The medium was removed and carefully washed with a phosphate buffered saline (PBS). The cells were detached from the culture dishes and collected in 5-ml round-bottom polystyrene tubes. The cells were stained with 25 μM of H_2DCF-DA in PBS for 30 minutes at 37 °C in a humidified atmosphere (95 % air-5 % CO_2). Fluorescence intensity was measured with BD FACS Canto™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Expression of MnSOD

Notochordal cells were plated onto poly (L-lysine)-coated 12-mm glass cover slips. The cells were fixed with 4 % paraformaldehyde for 15 minutes at room temperature and washed with PBS, pH 7.4. The cells were permeabilized by 0.2 % Triton X-100 in PBS for ten minutes. After incubation for ten minutes at room temperature with PBS containing 3 % BSA (blocking solution), cells were incubated with rabbit anti-MnSOD (Millpore Corp., Millipore Billerica, MA, USA) antibody in blocking solution for one hour at room temperature. After washing, fluorescence-conjugated Cy3-Donkey anti-rabbit antibody (Millpore Corp., Millipore Billerica, MA, USA) and Hoechst Nuclear HCS solution (Millpore Corp., Millipore Billerica, MA, USA) in blocking solution were added and the cells were incubated for one hour in the dark. After further washing steps, characteristic markers of organelle phenotype were determined by fluorescent microscope (Image Pro Plus) (Olympus).

Measurement of catalase level

Intracellular production of catalase by notochordal cells was measured with the Catalase Assay Kit (Cayman Chemical

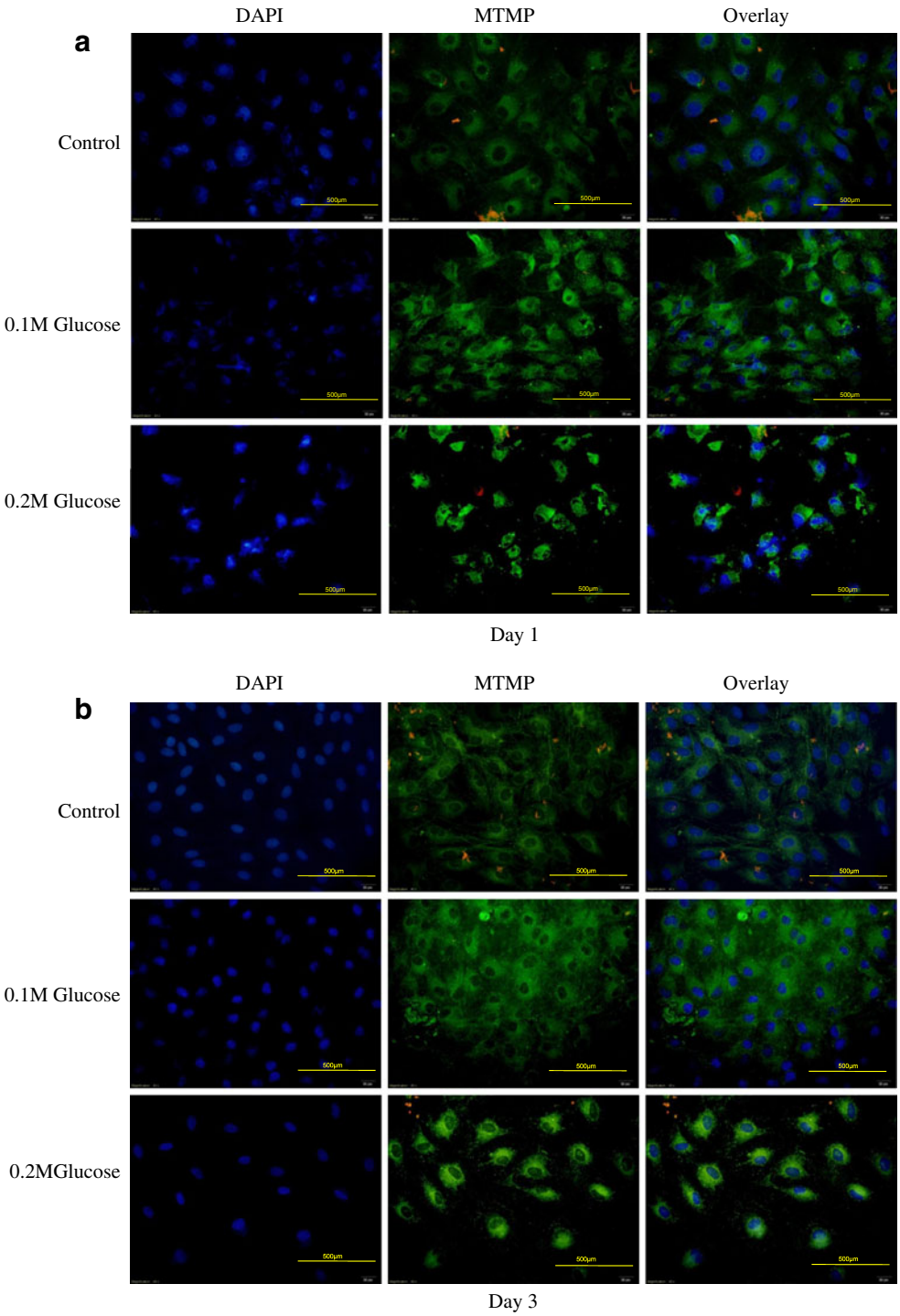

Fig. 1 Fluorescence microscope demonstrated an enhanced disruption of the mitochondrial transmembrane potential (*green color*) in rat notochordal cells treated with both high glucose concentrations one day (**a**) and three days (**b**), when compared to the control

Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. The cells were collected by centrifugation and plated in 12-well culture plates. The cells were sonicated on ice in 50 mM potassium phosphate, pH 7.0 containing 1 mM EDTA. Absorbance was measured at the 540 nm using a plate reader (Molecular Devices).

Expressions and activities of autophagy makers

Primary antibodies to beclin-1, light chain3-I (LC3-I) and LC3-II, and autophagy-related gene (Atg)3, 5, 7, and 12 were purchased from Cell Signaling Technology Inc., (Danvers, MA, USA) for western blot analysis of autophagy markers. In addition, antibody for loading control ß-actin was obtained

from SIGMA (Sigma, St. Louis, MO, USA). Expressions of beclin-1, LC3-I and -II, and Atg3, 5, 7, and 12 were determined by western blot analysis according to the manufacturer's instructions. ß-actin was used as an internal control for protein-loading.

Notochordal cells were washed with ice-cold PBS solution and lysed in PRO-PREP protein extraction solution (iNtRON Biotechnology, Inc., Korea). The cell lysates were centrifuged at 12,000 *g* for 15 minutes, and protein concentrations were measured using the Bicinchoninic Acid (BCA) (Thermo Fisher Scientific Inc., Pittsburgh PA, USA) method. Samples (50–70 μg of protein) were electrophoresed on 10–15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies, followed by second antibodies of HRP (horseradish peroxidase)-linked IgG (immunoglobulin G) (Bio-Rad, Richmond, CA, USA), and immunoreactive bands were visualized with the western blotting luminol reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Blots were quantified using Imaging Densitometer GF670 and Molecular Analyst software (Bio-Rad) three times in each sample, and the average of the three densities was used as the final density. The value of the density is presented as mean ± SD (arbitrary units).

Statistical analysis

All experiments were independently conducted three times, and results are expressed as mean and standard deviation. Statistical analysis was performed using a paired-samples t test. $P<0.05$ was considered significant.

Results

High glucose concentrations induce mitochondrial damage of notochordal cells

Fluorescence microscopy demonstrated an enhanced disruption (green color) of the mitochondrial transmembrane potential, which indicates mitochondrial damage and apoptosis, in rat notochordal cells treated with both high glucoses for one day (Fig. 1a) and three days (Fig. 1b), when compared to the control.

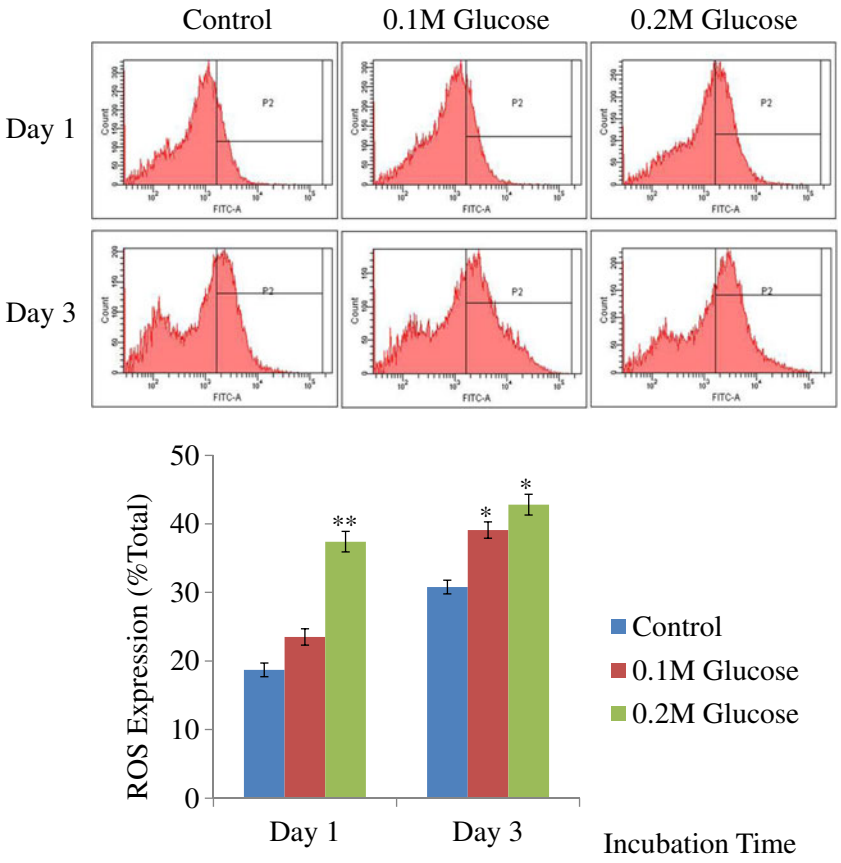

Fig. 2 Flow cytometry showed that each high glucose concentration enhanced ROS generation by rat notochordal cells in a dose- and time-dependent manner. $^{**}p<0.01$; $^{*}p<0.05$

High glucose concentrations increase the generation of ROS by rat notochordal cells

Flow cytometry showed that each high glucose concentration enhanced ROS generation by rat notochordal cells in a dose- and time-dependent manner (Fig. 2).

High glucose concentrations enhance MnSOD and catalase expression in rat notochordal cells

Immunofluorescence demonstrated that the two high glucose concentrations also enhanced compensatory expression (red color) of MnSOD in rat notochordal cells treated with both

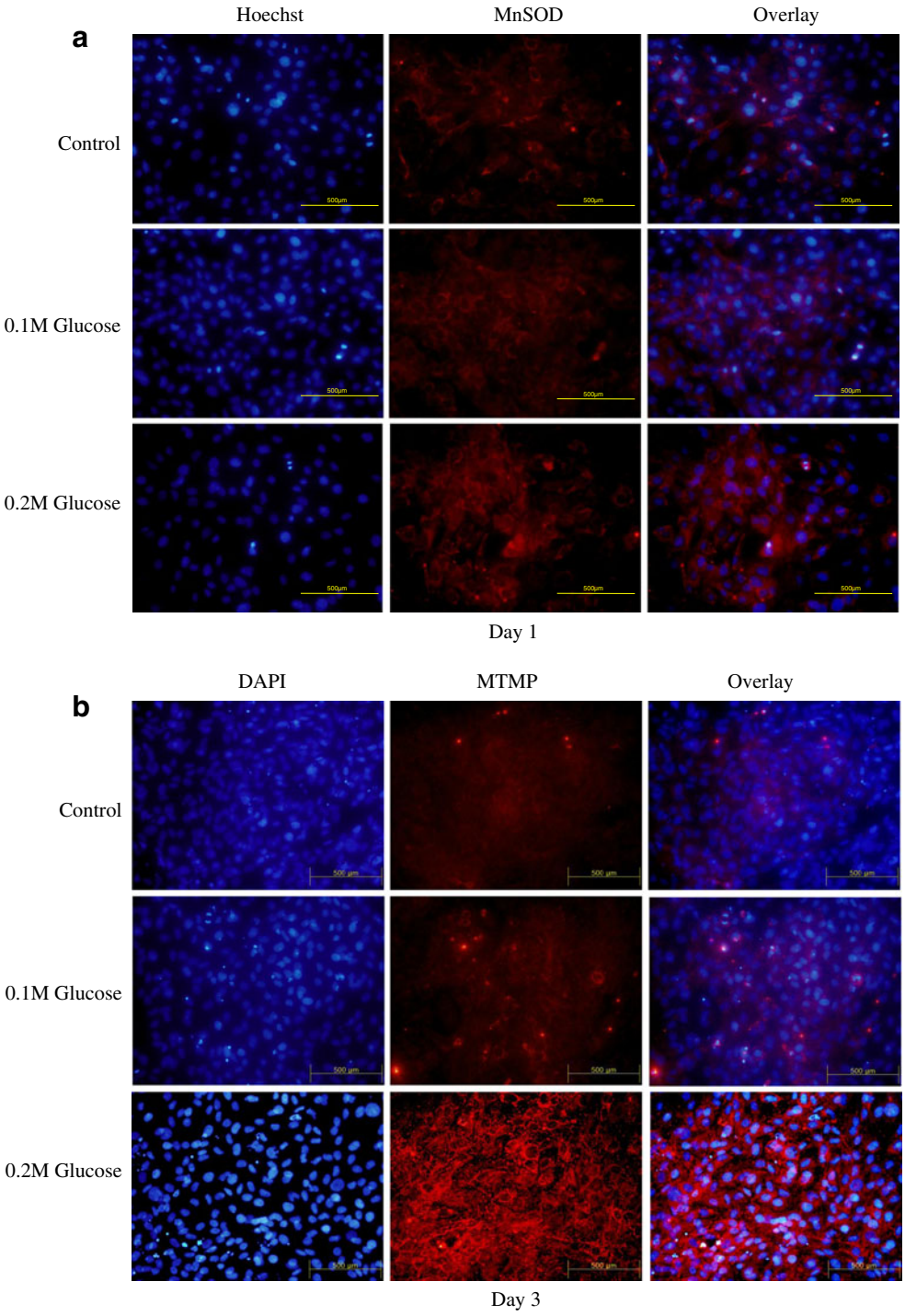

Fig. 3 Immunofluorescence demonstrated that the two high glucose concentrations enhanced compensatory expression of MnSOD (red-color) in rat notochordal cells treated with both high glucose concentrations for one day (**a**) and three days (**b**) compared to the control

high glucose concentrations for one day (Fig. 3a) and three days (Fig. 3b), when compared with the control. Western blot analysis showed a dose- and time-dependent increased expression of catalase in rat notochordal cells treated with both high glucose concentrations when compared to the control (Fig. 4).

High glucose concentrations promote rat notochordal cells' expression of autophagy markers

Western blot analysis showed a dose- and time-dependent increased expression of beclin-1, LC3-II, and Atg3, 5, 7, and 12 in notochordal cells treated with both high glucose concentrations when compared to the control (Fig. 5a and b). The ratio of LC3-II/LC3-I expression also increased in a dose- and time-dependent manner (Fig. 5c).

Discussion

This study shows that both high glucose concentrations induced mitochondrial damage, which was indicated by enhanced disruption of mitochondrial transmembrane potential, and increased generation of ROS by rat notochordal cells in a dose- and time-dependent manner. This increased oxidative stress caused enhanced compensatory expressions of antioxidants including MnSOD and catalase in a dose- and time-dependent manner. Finally, the proautophagic effects of high glucose were identified in the form of enhanced rat notochordal cells' expressions of beclin-I, LC3-II, and Agt3, 5, 7, and 12, in a dose- and time-dependent manner. The ratio of LC3-II/LC3-I expression also increased in a dose- and time-dependent manner. Overall, high glucose-induced oxidative stress promoted autophagy of rat notochordal cells through mitochondrial damage in a dose- and time-dependent manner. These results suggest that preventing the generation of oxidative stress might be a novel therapeutic target by which to prevent or delay IDD in patients with diabetes mellitus.

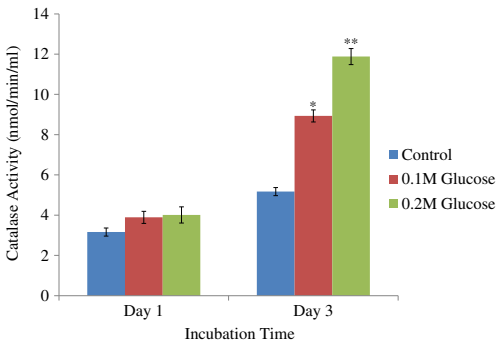

Fig. 4 Western blot analysis shows a dose- and time-dependent increased expression of catalase in rat notochordal cells treated with both high glucose concentrations compared to the control. $^{**}p<0.01$; $^{*}p<0.05$

Programmed cell death (PCD) refers apoptosis, autophagy and programmed necrosis, and is defined as the death of a cell in any pathological format, when mediated by an intracellular program [20–22]. These three forms of PCD may jointly control the fate of cells and balance cell death with survival of cells. Apoptosis, or type I PCD, is characterized by specific morphological and biochemical changes in dying cells, including cell shrinkage, nuclear condensation and fragmentation, dynamic membrane blebbing and loss of adhesion to neighbours or to the extracellular matrix. Previous studies have reported that excessive or premature apoptosis of intervertebral disc cells is associated with IDD [23, 24]. Moreover, diabetes mellitus has been reported to enhance the apoptosis of intervertebral disc cells and the expression of matrix-degrading and fibrotic enzymes [18, 19]. Autophagy (literally self-eating), or type II PCD, is a vital catabolic process by which cells engulf and breakdown intracellular proteins and organelles in the lysosome and repurpose the constituents for new biosynthesis [2–4]. It occurs continuously under normal conditions to remove and recycle damaged proteins and organelles as a method of quality control. Autophagy is a crucial mechanism that responds to either extra- or intracellular stress, and can result in cell survival under certain circumstances. However, over-activation of autophagy may result in autophagic cell death, which differs from apoptosis and programmed necrosis [4, 7–10]. Before this study, it had not yet been confirmed that high glucose-induced oxidative stress caused diabetes-related notochordal cells' autophagy. The authors therefore conducted the current study to evaluate the effects of high glucose concentrations on the induction of oxidative stress and autophagy through mitochondrial damage in rat notochordal cells.

The present study clearly demonstrated the effects of high glucose levels on the induction of oxidative stress and autophagy of rat notochordal cells through mitochondrial damage. Both high glucose concentrations significantly enhanced disruption of mitochondrial transmembrane potential of rat notochordal cells from culture on days one to three in a dose-dependent manner compared with 10 % FBS normal control. In addition, generation of ROS was significantly increased in each of the high glucose concentrations in a dose- and time-dependent manner when compared to the 10 % FBS normal control. It is well known that mitochondria, when damaged, release harmful ROS into the cytosol. Therefore, these findings suggest that diabetes mellitus enhances oxidative stress to rat notochordal cells via mitochondrial damage with dose- and time-dependent effects. Expressions of antioxidants such as MnSOD and catalase significantly increased in both high glucose concentrations compared to the 10 % FBS normal control. These findings suggest the possibility of a cycle wherein increased generation of ROS stress leads to compensatory production of antioxidants to neutralize oxidative stress.

The initiation and elongation of autophagy are mediated by ATGs and LC3 proteins [2–4]. Beclin-1 is part of the class III

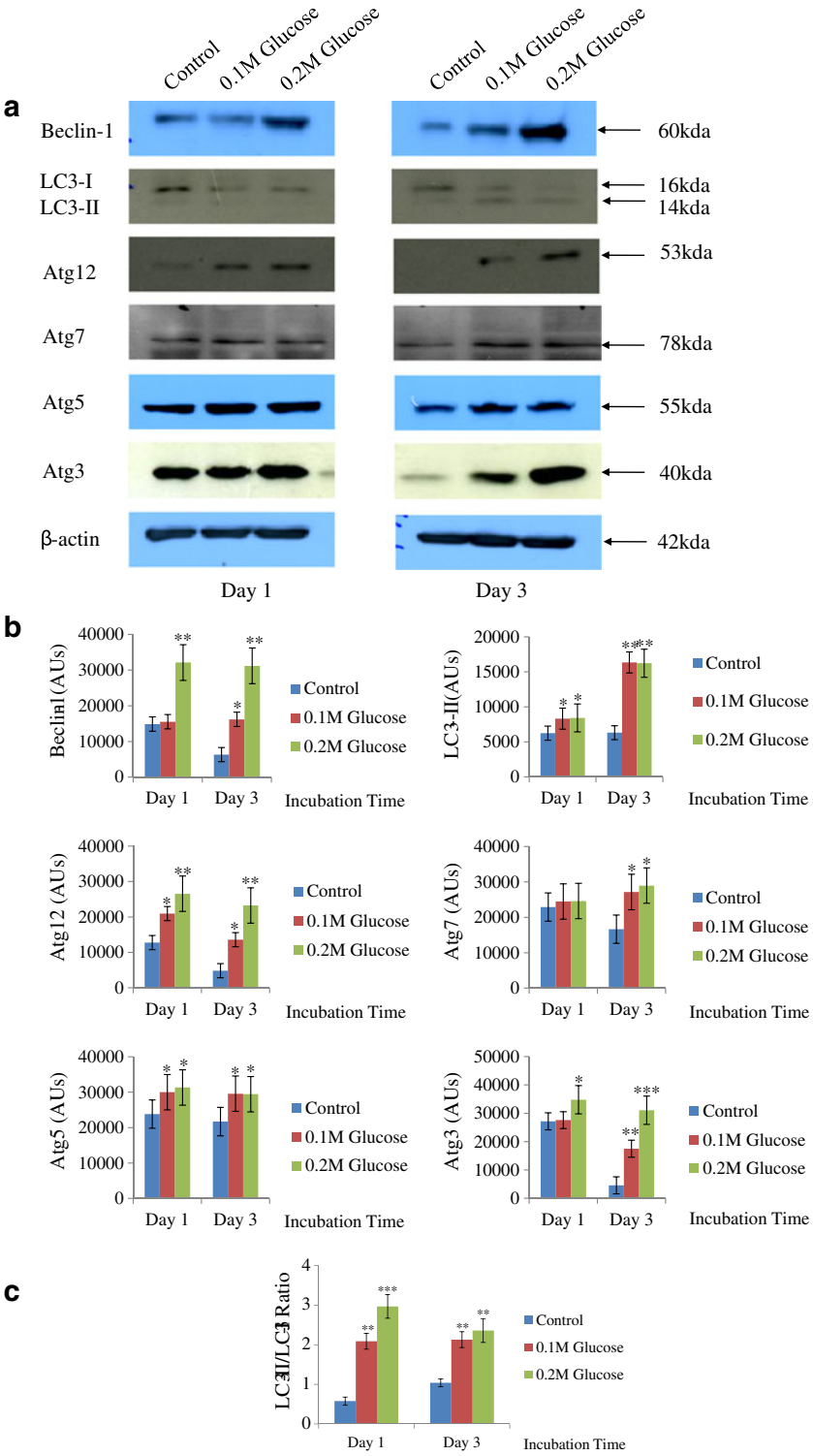

Fig. 5 Western blot analysis shows a dose- and time-dependent increased expression of beclin-1, LC3-II, and Atg3, 5, 7, and 12 in rat notochordal cells treated with both high glucose concentrations compared to the control (**a** and **b**). The ratio of LC3-II/LC3-I expression also increased in a dose- and time-dependent manner (**c**). ***$p<0.001$; **$p<0.01$; *$p<0.05$

PI3K complex that is required for the formation of the autophagic vesicle, and interference with beclin-1 prevents autophagy induction [5, 6]. This study demonstrates that both high glucose concentrations significantly increased the expressions of beclin-1, LC3, and ATG3, 5, 7 and 12 in notochordal cells from culture day one to three in a dose-dependent manner when compared to the 10 % FBS normal control. These findings suggest that high glucose-induced oxidative stress promotes autophagy of notochordal cells through mitochondrial damage in a dose- and time-dependent manner.

Notochordal cells form the notochord, which in turn contributes to the formation and maintenance of the nucleus pulposus of the intervertebral disc. After birth, the majority of notochordal cells gradually disappear in the nucleus pulposus.

In humans, notochordal cells are very rarely present after the age of ten years, and the nucleus pulposus transforms with time into a fibrocartilaginous nucleus pulposus [25]. With this transition, intervertebral disc degeneration begins. Therefore, the disappearance of notochordal cells in the nucleus pulposus is thought to be the starting point of intervertebral disc degeneration. A previous study reported that non-diabetic rat notochordal cells undergo apoptosis via the Fas type 2-mediated intrinsic (mitochondrial) pathway, and a regulated negative balance of notochordal cell proliferation against apoptosis is likely to involve the disappearance of notochordal cells [26]. Won et al. recently reported a cellular effect of diabetes mellitus on apoptosis of notochordal cells and intervertebral disc degeneration [18]. In their study using aged-matched diabetic and nondiabetic rats, Won et al. suggested that diabetes mellitus is associated with premature, excessive apoptosis of notochordal cells of the nucleus pulposus, accelerating the transition of a notochordal nucleus pulposus to a fibrocartilaginous nucleus pulposus. The premature phenotypic change of the nucleus pulposus in diabetic rats increased the expression of matrix degrading and fibrotic enzymes, resulting in rapid intervertebral disc degeneration and fibrosis [18, 19]. The present study showed that high glucose concentrations significantly increased the loss of rat notochordal cells by autophagy via oxidative stress and mitochondrial damage in a dose- and time-dependent manner. These previous studies and current results indicate that diabetes mellitus seems to enhance the disappearance of notochordal cells in the nucleus pulposus by autophagy as well as apoptosis, resulting in IDD in patients with diabetes mellitus.

In conclusion, this study demonstrates that high glucose-induced oxidative stress promotes autophagy through mitochondrial damage of rat notochordal cells in a dose- and time-dependent manner. These results suggest that preventing the generation of oxidative stress might be a novel therapeutic target by which to prevent or to delay IDD in patients with diabetes mellitus.

References

1. Srinvas V, Bohensky J, Zahm AM, Shapiro IM (2009) Autophagy in mineralizing tissues: microenvironmental perspectives. Cell Cycle 8:391–393
2. Hamasaki M, Yoshimori T (2010) Where do they come from? insights into autophagosome formation. FEBS Lett 584:1296–1301
3. Xie Z, Klionsky DJ (2007) Autophagosome formation: core machinery and adaptations. Nat Cell Biol 9:1102–1109
4. Wang SY, Yu QJ, Zhang RD, Liu B (2011) Core signaling pathways of survival/death in autophagy-related cancer networks. Int J Biochem Cell Biol 43:1263–1266
5. Furuya N, Yu J, Byfield M, Pattingre S, Levine B (2005) The evolutionarily conserved domain of Beclin 1 is required for Vps34 binding, autophagy and tumor suppressor function. Autophagy 1:46–52
6. He C, Zhu H, Li H, Zou MH, Xie Z (2013) Dissociation of Bcl-2-Beclin 1 complex by activated AMPK enhances cardiac autophagy and protects against cardiomyocyte apoptosis in diabetes. Diabetes 62:1270–1281
7. Chen ZF, Li YB, Han JY, Wang J, Yin JJ, Li JB, Tian H (2011) The double-edged effect of autophagy in pancreatic beta cells and diabetes. Autophagy 7:12–16
8. Carames B, Taniguchi N, Otsuki S, Blanco FJ, Lotz M (2010) Autophagy is a protective mechanism in normal cartilage and its aging-related loss is linked with cell death and osteoarthritis. Arthritis Rheum 62:791–801
9. Murrow L, Debnath J (2013) Autophagy as a stress-response and quality-control mechanism: implications for cell injury and human disease. Annu Rev Pathol 8:105–137
10. Jing K, Lim K (2012) Why is autophagy important in human diseases. Exp Mol Med 44:69–72
11. Victor VM, Rocha M, Herance R, Hernandez-Mijares A (2011) Oxidative stress and mitochondrial dysfunction in type 2 diabetes. Curr Pharm Des 17:3947–3958
12. Baynes JW (1991) Role of oxidative stress in development of complications in diabetes. Diabetes 40:405–412
13. Jung HS, Lee MS (2010) Role of autophagy in diabetes and mitochondria. Ann NY Acad Sci 1201:79–83
14. Nishikawa T, Araki E (2007) Impact of mitochondrial ROS production in the pathogenesis of diabetes mellitus and its complications. Antioxid Redox Signal 9:343–353
15. Dorn GW 2nd (2010) mechanism of non-apoptotic programmed cell death in diabetes and heart failure. Cell Cycle 9:3442–3448
16. Kume S, Thomas MC, Koya D (2012) Nutrient sensing, autophagy, and diabetic nephropathy. Diabetes 61:23–29
17. Li L, Ishdorj G, Gibson SB (2012) Reactive oxygen species regulation of autophagy in cancer: implications for cancer treatment. Free Radic Biol Med 53:1399–1410
18. Won HY, Park JB, Park EY, Riew KD (2009) Effect of hyperglycemia on apoptosis of notochordal cells and intervertebral disc degeneration in diabetic rats. J Neurosurg Spine 11:741–748
19. Park EY, Park JB (2013) Dose- and time-dependent effect of high glucose concentration on viability of notochordal cells and expression of matrix degrading and fibrotic enzymes. Int Orthop 37:1179–1186
20. Ouyang L, Shi Z, Zhao S, Wang FT, Zhou TT, Liu B, Bao JK (2012) Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. Cell Prolif 45:487–498
21. Mughal W, Dhingra R, Kirshenbaum LA (2012) Striking a balance: Autophagy, apoptosis, and necrosis in a normal and failing heart. Curr Hypertens Rep 14:540–547
22. Almonte-Becerril M, Navarro-Garcia F, Gonzales-Robles A, Vega-Lopez MA, Lavalle C, Kouri JB (2010) Cell death of chondrocytes is a combination between apoptosis and autophagy during the pathogenesis of osteoarthritis within an experimental model. Apoptosis 15:631–638
23. Park JB, Lee JK, Park SJ, Kim KW, Riew KD (2005) Mitochondrial involvement in Fas-mediated apoptosis of human lumbar disc cells. J Bone Joint Surg Am 87:1338–1342
24. Park JB, Kim KW, Han CW, Chang H (2001) Expression of Fas receptor on disc cells in herniated lumbar disc tissue. Spine 26:142–146
25. Kim KW, Lim TH, Kim JG, Jeong ST, Masuda K, An HS (2003) The origin of chondrocytes in the nucleus pulposus and histologic findings associated with the transition of a notochordal nucleus pulposus to a fibrocartilaginous nucleus pulposus in intact rabbit intervertebral discs. Spine 28(Phila Pa 1976):982–990
26. Kim KW, Kim YS, Ha KY, Woo YK, Park JB, Park WS, An HS (2005) An autocrine or paracrine Fas-mediated counterattack: a potential mechanism for apoptosis of notochordal cells in intact rat nucleus pulposus. Spine 30(Phila Pa 1976):1247–1251