Low-calorie sweetener D-psicose promotes hydrogen peroxide-mediated apoptosis in C2C12 myogenic cells favoring skeletal muscle cell injury

ZHEN-JIE WEI1,2*, LU SUN3*, YU-LIN LI2*, JIBRAN SUALEH MUHAMMAD4, YING WANG1, QIAN-WEN FENG2, YAN-ZHUO ZHANG1, HIDEKUNI INADERA2, ZHENG-GUO CUI1,2,5 and CHENG-AI WU1

1Department of Molecular Orthopedics, Beijing Research Institute of Traumatology and Orthopedics, Beijing Jishuitan Hospital, Beijing 100035, P.R. China; 2Department of Public Health, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan; 3Department of Pediatric Cardiology, Heart Center, Guangzhou Women and Children’s Medical Center, Guangzhou Medical University, Guangzhou, Guangdong 510623, P.R. China; 4Department of Basic Medical Sciences, College of Medicine, University of Sharjah, Sharjah 27272, United Arab Emirates; 5Department of Environmental Health, School of Medical Science, University of Fukui, Fukui 910-1193, Japan

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Abstract. Diet and exercise are the most effective approaches used to induce weight loss. D-psicose is a low-calorie sweetener that has been shown to reduce weight in obese individuals. However, the effect of D-psicose on muscle cells under oxidative stress, which is produced during exercise, requires further investigation. The present study aimed to determine the effects of D-psicose on C2C12 myogenic cells in vitro. Hydrogen peroxide (H2O2) was used to stimulate the generation of intracellular reactive oxygen species (ROS) in muscle cells to mimic exercise conditions. Cell viability was analyzed using a MTT assay and flow cytometry was used to analyze the levels of apoptosis, mitochondrial membrane potential (MMP), the generation of ROS and the cell cycle distribution following treatment. Furthermore, protein expression levels were analyzed using western blotting and cell proliferation was determined using a colony formation assay. The results of the present study revealed that D-psicose alone exerted no toxicity on C2C12 mouse myogenic cells. However, in the presence of low-dose (100 µM) H2O2-induced ROS, D-psicose induced C2C12 cell injury and significantly decreased C2C12 cell viability in a dose-dependent manner. In addition, the levels of apoptosis and the generation of ROS increased, while the MMP decreased. MAPK family molecules were also activated in a dose-dependent manner following treatment. Notably, the combined treatment induced G2/M phase arrest and reduced the proliferation of C2C12 cells. In conclusion, the findings of the present study suggested that D-psicose may induce toxic effects on muscle cells in a simulated exercise situation by increasing ROS levels, activating the MAPK signaling pathway and disrupting the MMP.

Introduction

Over the past four decades, the prevalence of obesity has tripled worldwide (1). As the number of individuals that are overweight and obese increases, the consumption of low-calorie sweeteners has also gradually increased each year (2). Although low-calorie synthetic sweeteners, such as aspartame and saccharine, may have a beneficial effect on weight loss, several studies have reported adverse effects (3). Therefore, nature-derived sugar substitutes with low calorific contents, such as D-psicose, have been developed.

D-psicose, also known as D-allulose, is produced in small quantities in various fruits. It is an epimer of fructose that can be enzymatically produced using D-psicose 3-epimerase (4). In addition to its anti-obesity effects, D-psicose has also been found to inhibit cancer cell proliferation by inducing apoptosis and cell cycle arrest (5-7). Since the purpose of using low-calorie sweeteners is to help lose weight, it is likely that individuals will also be regularly exercising while consuming D-psicose. However, the effect of the chronic consumption
of D-psicose on exercising muscle tissue remains unclear. A previous in vivo study reported that the oral administration of D-psicose was well absorbed and distributed throughout the body; the highest accumulation was found in the liver and kidneys, followed by the lungs, spleen and skeletal muscle tissue (8). Skeletal muscles comprise >40% of body organ systems. The muscles in those who exercise regularly are highly metabolically active and use the majority of the simple sugars available. Thus, the use of D-psicose by an obese individual who regularly exercises may lead to muscle cells being highly exposed to this natural sugar. Therefore, the effect of D-psicose on muscle cells under exercise-induced oxidative stress requires further investigation.

During exercise, the rate of muscle contractile activity exponentially increases, which leads to overactive mitochondria in muscle cells and the production of reactive oxygen species (ROS), such as superoxide free radicals (9). Certain types of free radicals have been detected in muscle tissue and increased free-radical activity has been shown to lead to extensive muscle damage. The produced reactive species can positively or negatively modulate muscle cells (10). Previous studies have reported that D-psicose upregulated the expression levels of CDKs, leading to cell cycle arrest and cell cycle progression and cell proliferation (15). Notably, several previous studies have reported that D-psicose upregulated the expression levels of CDKs, leading to cell cycle arrest and apoptosis in several cell types (5-7).

The present study aimed to determine the effects of D-psicose on C2C12 myogenic cells. Low dose H₂O₂ was used to mimic the generation of ROS during exercise and to determine how D-psicose affected skeletal muscle cells under oxidative stress-induced conditions, such as exercise. The results revealed that D-psicose, in the presence of physiological concentrations of H₂O₂, induced the generation of ROS, triggered cell cycle arrest and initiated apoptosis in C2C12 myogenic cells. To the best of our knowledge, this was the first study to demonstrate the toxic effects of D-psicose on muscle cells treated with H₂O₂, which were suggested to occur via the MAPK signaling pathway.

Materials and methods

Chemicals. D-psicose, H₂O₂, N-acetylcysteine (NAC), JNK inhibitor VIII and SB203580 were all purchased from Sigma-Aldrich (Merck KGaA).

Cell culture and treatment. C2C12 cells were obtained from the Human Sciences Research Resources Bank (Japan Human Sciences Foundation, Tokyo, Japan). Cells were cultured in low glucose DMEM (Thermo Fisher Scientific, Inc.), supplemented with 10% heat-inactivated FBS (Gibco; Thermo Fisher Scientific, Inc.) and maintained at 37°C in a humidified atmosphere with 5% CO₂. For each experiment, cells were seeded in a 6-well plate and pretreated with 1, 2 or 5 mM D-psicose for 3 h and then with 100 μM H₂O₂ for 2 h, after which the medium was changed, followed by incubation for 19 h.

MTT assay. An MTT assay was used to evaluate cell viability. Briefly, cells were seeded into 96-well plates at a density of 3x10⁵ cells/well and incubated overnight. Following the incubation, 20 μl sterile MTT dye (5 mg/ml) was added to each well and incubated for another 3 h at 37°C. After removal of the medium, 100 μl DMSO was added to each well and incubated for a further 10 min. The absorbance was measured at a wavelength of 570 nm using a microplate reader (16). Eight replicate wells were used for each concentration.

Flow cytometric analysis of apoptosis. Cells were stained with Annexin V-FITC and propidium iodide (PI; cat. no. BMSS000FL-300; Thermo Fisher Scientific, Inc.) as previously described (17). Apoptotic cells were subsequently analyzed using flow cytometry (BD FACSCanto II flow cytometer and FACSDiva ver. 6.1; BD Biosciences) according to the manufacturer’s protocol.

Colony formation assay. Cells (1x10⁵/well) were seeded into 6-well plates and cultured in the indicated media for 10-15 days. Subsequently, the media was removed, cells were washed twice in PBS, fixed with 4% paraformaldehyde for 1 h at room temperature and stained with crystal violet for 40 min at room temperature. Plates were then thoroughly washed with water and air-dried.

Cell cycle distribution analysis. Cells were harvested after 24 h of treatment and centrifuged at 200 x g for 5 min at room temperature. Cell pellets were suspended in 100 μl PBS prior to being fixed with 75% (v/v) cold ethanol for 2 h at room temperature and then stained with a PI solution containing DNase-free RNase A for 30 min at room temperature in the dark. Analysis was performed using a flow cytometer according to the manufacturer’s protocol.

Measurement of intracellular ROS levels. Intracellular ROS levels were detected using dihydroethidium (DHE; Molecular Probes; Thermo Fisher Scientific, Inc.) using flow cytometry. DHE produces blue fluorescence in the cytosol until it is oxidized by superoxide to 2-hydroxyethidium, which then intercalates within cellular DNA and stains the nucleus a bright fluorescent red. Following incubation with 4 μM DHE for 15 min at 37°C, the cells were washed twice with PBS and the intracellular ROS levels (%) were analyzed using flow cytometry.

Measurement of the mitochondrial membrane potential (MMP). Following treatment, the cells were collected and treated with 10 nM tetramethylrhodamine methyl ester perchlorate (TMRM;
Molecular Probes; Thermo Fisher Scientific, Inc.) in 1 ml FBS (1%) diluted in PBS for 15 min at 37°C. TMRM is a cationic fluorophore widely used for staining cellular mitochondria and the mitochondrial matrix. The percentage of cells with potential MMP loss was analyzed by flow cytometry gated for red TMRM fluorescence, using an excitation wavelength of 488 nm and an emission wavelength of 575 nm (18).

**Western blotting.** Cells were harvested, washed with PBS and total protein was extracted using RIPA lysis buffer (150 mM NaCl, 1% Triton X-100 (v/v), 1% sodium deoxycholate, 0.1% SDS, 1 µg/ml each of aprotinin, pepstatin and leupeptin, 1 mM EGTA, 50 mM Tris–HCl; pH 7.5) for 20 min on ice. Following a brief sonication, lysates were centrifuged at 13,000 x g for 10 min at 4°C, the supernatant was collected and the protein contents in the supernatant were measured using a Coomassie (Bradford) Protein Assay kit (Thermo Fisher Scientific, Inc.). An equal amount of protein (10 µg) was loaded per lane and separated via SDS-PAGE. The separated proteins were subsequently transferred onto PVDF membranes and blocked with 5% skimmed milk in TBS-Tween 20 (150 mM NaCl, 50 mM Tris, 0.1% Tween-20; pH 7.5) for 1 h at room temperature. The membrane was then incubated overnight at 4°C with the following primary antibodies: Anti-MCL1 apoptosis regulator BCL2 family member (Mcl-1; cat. no. CST68542; 1:1,000), anti-cleaved caspase-3 (cat. no. CST9661; 1:1,000), anti-JNK (cat. no. CST9258; 1:1,000), anti-phosphorylated (p)-JNK (cat. no.CST4668; 1:1,000), anti-ERK1/2 (cat.no.CST4695; 1:1,000), anti-p-ERK1/2 (cat. no. CST4370; 1:2,000), anti-p38 (cat. no. CST8690; 1:1,000), anti-p-p38 (cat. no. CST4511; 1:1,000), anti-p-checkpoint kinase 1 (Chk1; cat. no. CST2348; 1:1,000), anti-p-DNA polymerase δ 1, catalytic subunit (CDC2; cat. no. CST4539; 1:1,000), anti-sirtuin 3 (SIRT3; cat. no. CST5490; 1:1,000) (all Cell Signaling Technology, Inc.), anti-Bax (cat. no. ab32503; 1:5,000), anti-cleaved-poly(ADP-ribose) polymerase 1 (PARP1; cat. no. ab32064; 1:1,000; both Abcam), anti-cell division cycle 25 C (CDC25C; cat. no. sc327; 1:1,000), anti-superoxide dismutase 2 (SOD2; cat. no. sc30080; 1:1,000), anti-Bcl-2 (cat. no. sc7382; 1:400) and anti-β-actin (cat. no. sc8432; 1:1,000; all Santa Cruz Biotechnology, Inc.).

Following the primary antibody incubation, the membranes were incubated for 2 h at room temperature with the following secondary antibodies: Anti-rabbit IgG, HRP-linked Antibody (cat. no. CST7074; 1:3,000) and anti-mouse IgG, HRP-linked Antibody (cat. no. CST7076; 1:2,000; both Cell Signaling Technology, Inc.). Protein bands were visualized using ECL (Amersham; Cytiva) according to the manufacturer’s protocol (19). Densitometric analysis was performed using ImageJ software 1.50i (National Institutes of Health).

**Statistical analysis.** All experiments are representative of three independent biological replicates. Data are presented as the mean ± SD. Multiple comparisons were performed using a one-way or two-way ANOVA with a Tukey’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

*D-psicose treatment exerts no cytotoxic effects on resting muscle cells.* D-psicose is a ketohexose monosaccharide with the following molecular formula: C₆H₁₂O₆. It is a C₃ epimer of fructose that was first derived from the antibiotic psicofuranine, which contains one ketone group that acts as a reducing agent (4). The three-dimensional structure produces a white, odorless powder that is soluble in water (Fig. 1A and B). The present study first investigated the effect of D-psicose on C2C12 myogenic cells in resting conditions (without oxidative stress). The results demonstrated that increasing concentrations of D-psicose (0-5 mM) exerted no cytotoxic effects on resting C2C12 cells at 24 or 48 h post-treatment (Fig. 1C), which suggested the potential safe application of D-psicose as a sugar replacement in daily food products.

*D-psicose exacerbates H₂O₂-induced cell damage in C2C12 cells.* To mimic exercise-induced oxidative stress in muscle cells, C2C12 cells were treated with H₂O₂. The results revealed that as the concentration of H₂O₂ increased, the viability of C2C12 cells decreased; ~10% of the cells had decreased viability following 100 µM H₂O₂ treatment and only 60% of cells remained viable following 500 µM H₂O₂ treatment (Fig. 2A). Thus, 100 µM H₂O₂ was selected as the optimal concentration to act as an exercise mimic in further experiments.

C2C12 cells were pre-incubated with D-psicose for 3 h and then treated with H₂O₂ for 2 h, after which the cell medium was replaced with fresh cell medium, and cells were incubated for a further 19 h. Cell viability analysis revealed a dose-dependent decrease in cell viability in cells treated with D-psicose in the presence of H₂O₂. Notably, viability was lost in >50% of cells following 5 mM D-psicose treatment (Fig. 2B).

Subsequently, whether H₂O₂ induced apoptosis in D-psicose pretreated cells was determined (Fig. 2C and D).
Figure 2. D-psicose enhances H$_2$O$_2$-induced C2C12 cell damage. (A) C2C12 cells were pretreated with 0-500 µM H$_2$O$_2$ for 2 h, after which the medium was changed. Following incubation for 24 h, a MTT assay was used to analyze cell viability. The concentration of 100 µM H$_2$O$_2$ was determined to be a moderate inducer of oxidative stress. C2C12 cells were pretreated with 1, 2 or 5 mM D-psicose for 3 h and then with 100 µM H$_2$O$_2$ for 2 h, after which the medium was changed. Following incubation for 19 h, (B) an MTT assay, (C) Annexin V/propidium iodide staining, (D) flow cytometry, (J and K) tetramethylrhodamine methyl ester perchlorate staining and (E-i) western blotting were used to analyze cell viability, cell apoptosis, MMP loss and the expression levels of apoptosis-related proteins. Data are presented as the mean ± SEM of independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. nT; *P<0.05, **P<0.01 and ***P<0.001 vs. H$_2$O$_2$ (n=3). H$_2$O$_2$, hydrogen peroxide; MMP, mitochondrial membrane potential; Mcl-1, MCL1 apoptosis regulator BCL2 family member; PARP-1, anti-cleaved-poly(ADP-ribose) polymerase 1; NT, No treatment.
To investigate the effect of D-psicose on the apoptotic pathway, expression levels of proteins associated with apoptosis were analyzed using western blotting. D-psicose was found to potentiate H₂O₂-induced apoptosis by downregulating the expression levels of the anti-apoptotic proteins, Bcl-2 and Mcl-1, and upregulating the expression levels of the proapoptotic protein Bax (Fig. 2e-i). A concomitant increase in the cleavage of caspase-3 and PARP1 was also observed, suggesting that D-psicose initiated caspase-mediated apoptosis. When cells are under immense oxidative stress, mitochondria lose their membrane integrity and exercising muscles rely on mitochondria for the majority of their energy production. Thus, the effects of D-psicose on mitochondrial function were investigated. The treatment with H₂O₂ or D-psicose alone did not affect the mitochondria of muscle cells; however, in the presence of D-psicose pretreatment, H₂O₂ treatment significantly decreased the MMP in C2C12 cells (Fig. 2J and K).
D-psicose exacerbates H$_2$O$_2$-induced apoptosis in C2C12 cells through the MAPK signaling pathway. Exercising is an intermittent form of cellular stress that was discovered to activate proteins of the MAPK signaling pathways in the skeletal muscle of rats (20). In addition, the H$_2$O$_2$-induced upregulation of ERK, p38 and JNK expression was reported to regulate cell death in C2C12 cells (21). To investigate the role of MAPKs in the damaging effect of D-psicose in exercising skeletal muscle cells, the protein expression levels of ERK, p38 and JNK were analyzed in H$_2$O$_2$-stimulated C2C12 cells.
The expression levels of p-JNK and p-p38 were significantly upregulated following the pretreatment with increasing concentrations of D-psicose in H2O2-treated C2C12 cells; however, the expression levels of p-ERK were downregulated (Fig. 3 A-D). These data suggested that JNK and p38 may have a role in the regulation of D-psicose-induced apoptosis in muscle cells under oxidative stress conditions.

To validate these findings, muscle cells were exposed to JNK and p38 inhibitors (JNK inhibitor VIII and SB203580, respectively). Following the pre-incubation of the C2C12 cell culture with JNK inhibitor VIII or SB203580, followed by treatment with D-psicose and H2O2, the expression levels of p-JNK and p38 were downregulated. Concurrently, both of the inhibitors also reversed D-psicose-induced apoptotic effects by upregulating...
The present study aimed to determine the effect of D-psicose on ROS generation and H$_2$O$_2$-induced apoptosis in C2C12 myogenic cells. H$_2$O$_2$ is a widely used method to establish exercise-induced oxidative stress in cells (11,12,22). The present results revealed that the treatment of C2C12 cells with D-psicose under oxidative stress conditions elevated the levels of ROS, decreased the MMP and apoptosis, and induced cell cycle arrest. Moreover, the lethal effects of D-psicose on exercising muscle cells were found to be mediated via the MAPK family molecules, p-JNK and p-p38 (Fig. 6). Notably, the observed effects were reversed following treatment with the ROS inhibitor, NAC, which is consistent with the findings of a previous study (24).

Oxidative stress is the production of excessive reactive oxidants that exceeds the cellular antioxidant capability. In 1992, it was reported that contracting rodent skeletal muscle released superoxide radicals into the interstitial spaces (25). Bailey et al (26) also demonstrated that exercise resulted in a greater production and accumulation of intramuscular free radicals in human skeletal muscle cells. ROS produced in contracting skeletal muscle facilitates the strength of the muscle contraction; however, if ROS concentrations exceed a certain level, it reduces force generation and causes fatigue (27). The findings of the present study revealed that low doses of H$_2$O$_2$ induced oxidative stress in muscle cells, which may be due to exercise-induced ROS generation. However, the concomitant exposure to D-psicose further increased the production of ROS, leading to cell death. On the other hand, pretreatment with NAC reversed the increased production of ROS, returning the levels of ROS to almost homeostatic levels, thereby providing a protective effect. These results were consistent with previous studies, which showed that antioxidant infusion in humans during voluntary exercise substantially enhanced performance during prolonged exercise (28,29).

The balance between antioxidants and pro-oxidants is crucial for the survival of aerobic organisms. A previous experiment in rats demonstrated that exercise promoted the expression of...
and activity of SOD2 in skeletal muscle mitochondria, which subsequently maintained cellular oxidant-antioxidant homeostasis (30). Therefore, as the exercise-induced upregulation of SOD2 in skeletal muscle served a key role in the prevention of superoxide accumulation, it could be hypothesized that the loss of SOD2 may have devastating effects on muscle cell survival. SIRT3 was discovered to regulate SOD2 via deacetylation and activation of FOXO3a, and SOD2 can catalyze the neutralization process involving the conversion of superoxide into water and hydrogen peroxide (23,31). The results of the present study revealed that the treatment with D-psicose alone, in addition to in the presence of H2O2, exerted inhibitory effects on the expression levels of SIRT3 and SOD2.

D-psicose was found to suppress post-prandial serum glucose levels and reduce the accumulation of body fat, which indicates its beneficial effects on obesity-related metabolic disturbances (8). In the past, this type of sugar supplement was used in tabletop packets in cafés and restaurants. Currently, these sugars are added to everyday food products to promote the sales of food items as healthy alternatives. In both children and adults, the consumption of low-calorie sweeteners has significantly increased, primarily due to an increased awareness of their non-nutritive value and potential effects in weight loss (32). Hence, future studies should aim to enhance the existing knowledge on the metabolic and health effects of low-calorie sweeteners. A long-term study on D-psicose toxicity in rats revealed that the final body weight and weight gain in D-psicose-fed animals were significantly decreased compared with the control group (33). On the one hand, these findings may be considered as beneficial for weight loss; however, D-psicose may reduce the body weight by decreasing the number of muscle cells. Yagi and Matsuo (33) investigated the long-term (18 months) toxicity of D-psicose in rats and found a significant increase in organ weight of the liver, kidneys, brain, lungs and pancreas, which suggested that the accumulation of fat, and that muscle proteins may be broken down to promote toxic effects. The present data suggested that the treatment with D-psicose was safe for muscle cells; however, under stressful conditions, D-psicose could exert deleterious effects on the survival of the muscle cells.

Previous studies have shown that D-psicose increased G2/M phase cell cycle arrest and decreased the population of liver cells in the S phase (5). Cell cycle progression is tightly regulated by a complex network of positive and negative cell cycle regulatory molecules, such as cyclins and CDKs. CDC25C is involved in the DNA damage checkpoints by activating CDK complexes that drive the cell cycle and is a key mediator of cell cycle progression (34). The current findings demonstrated that the treatment with D-psicose in H2O2-stimulated muscle cells led to the accumulation of cells in the sub-G1 phase and the downregulation of the expression levels of the cell cycle-regulating protein, CDC25C.

In conclusion, the findings of the present study suggested that under physiological oxidative stress conditions, D-psicose may exert negative effects on muscle cells by promoting the production of ROS and triggering cell cycle arrest and apoptosis. The results further indicated that the negative effects of D-psicose on exercising muscle cells may be mediated by MAPK and may be partly due to slow cell cycle progression and the failure to repair DNA damage, which ultimately leads to apoptosis.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
ZW and LS designed the study. ZW and YL performed the experiments and collected the data. ZC and CW confirm the authenticity of all the raw data. ZW interpreted the data and experiments and wrote the manuscript with help from ZC and CW. ZC and CW wrote the manuscript with help from ZC and CW. ZC and CW were responsible for funding acquisition. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

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Not applicable.

Competing interests
The authors declare that they have no competing interests.

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