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The protective effect of MT-α-glucan against streptozotocin (STZ)-induced NIT-1 pancreatic β-cell damage

Lei Hong[a,*], Wang Qin[a], Guo Shuzhen[a], Han Juncheng[a], Sun Hanju[a], Zhang Xiaoxiang[a], Wu Wutong[b]

[a] School of Biology and Food Engineering, Hefei University of Technology, Hefei 230009, China
[b] School of Life Science and Technology, China Pharmaceutical University, Nanjing 210009, China

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ABSTRACT

The protective effect of an alpha-glucan (designated here as MT-α-glucan) from fruit body of maitake (*Grifola frondosa*) on NIT-1 pancreatic β-cells damaged by streptozotocin (STZ) in vitro was investigated. The cell viability, insulin secretion, the activity of superoxide dismutase (SOD), glutathione peroxidase (GSHpx) and the content of reduced glutathione (GSH) increased significantly when the cells were incubated with MT-α-glucan (400, 200 μg ml^{-1}). The content of malondialdehyde (MDA), nitric oxide (NO) production, and the activity of NO synthase (NOS), inducible NOS (iNOS) decreased significantly when the cells were incubated with MT-α-glucan. The destructive changes of NIT-1 cells ameliorated when incubated with MT-α-glucan under microscopic observation. These data suggested that MT-α-glucan had obvious protective effect on NIT-1 pancreatic β-cells damaged by STZ, which might be related to its effects of decreasing levels of β-cell-destroying factors such as oxidative stress and NO synthesis.

1. Introduction

Maitake mushrooms (*Grifola frondosa*) belong to Basidiomycetes in fungi and have been praised and consumed by Chinese people for hundreds of years as of their enticing tastes. Moreover, the medicinal properties of maitake have been claimed for years and some of them have been demonstrated scientifically and experimentally. For instance, maitake has been shown to have antitumor effect (Liu, Chen, & Wu, 2005), immune regulatory activity (Inoue, Kodoma, & Nanba, 2002), anti-hyperliposis (Kubo, 1997), anti-common and specific infections effects such as hepatitis (Kubo & Nanba, 1998; Ooi, 1996) and AIDS/HIV (Nanba, Kodama, Schar, & Turner, 2000).

Previous studies have shown that ingesting maitake mushrooms, or some of its extracts, influenced glucose/lipid metabolism and had anti-diabetic effect (Kubo, 1994, 1997). But studies on its active part and mechanism of action have not been carried out. Based on previous studies, a new kind of alpha-glucan extracted and purified from the fruit body of maitake, designated here as MT-α-glucan, was prepared in our laboratory. Previous studies in our laboratory have shown that MT-α-glucan has hypoglycemic activity in KK-Ay mice (Lei, Ma, & Wu, 2007) and experimental type 2 diabetes mice (Lei et al., 2012), by ameliorating peripheral insulin resistance and protecting pancreatic islets in vivo. The present study was therefore designed to explore the protective effect of MT-α-glucan on NIT-1 pancreatic β-cells damaged by streptozotocin (STZ) in vitro. Moreover, its mechanisms of action concerning protection of pancreatic β cells from destruction by oxidative stress and nitric oxide (NO) in vitro were also investigated.

2. Materials and methods

2.1. Preparation, purity and structure of MT-α-glucan

The procedure of preparation of MT-α-glucan and its purity and primary structure were studied in our laboratory previously and had been reported (Lei et al., 2007; Ma et al., 2007), which were described briefly below.

MT-α-glucan was extracted and purified from the fruit body of maitake (*G. frondosa*), manufactured in Zhejiang province and identified by China Pharmaceutical University. Dried powdered fruit bodies of maitake were refluxed with diethylether and ethylalcohol mixture at 70 °C for 6 h, and then centrifuged and the residue collected and extracted by distilled water at 121 °C for 30 min. The extract was centrifuged and the supernatant was collected and precipitated in 95% ethyl alcohol at 4 °C for 12 h. This was followed by centrifugation and the precipitate was collected and re-dissolved in distilled water, then fractionated by DEAE Sepharose Fast Flow chromatography. The fraction was collected, concentrated and

* Corresponding author at: School of Biology and food engineering, Hefei University of Technology, Tunxi Road 193#, Hefei, Anhui 230009, China.
Tel.: +86 551 2901395; fax: +86 551 2901395.
E-mail address: leihong56@163.com (L. Hong).

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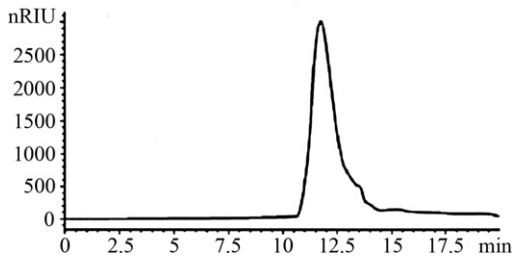

Fig. 1. HPGPC chromatography of MT-α-glucan.

freeze dried, and was designated as MT-α-glucan. MT-α-glucan was dissolved in DMEM medium and diluted to the concentration needed.

The homogeneity of the compound MT-α-glucan was estimated by high-performance gel-permeation chromatography (HPGPC). HPGPC was performed using Agilent 1100 series HPLC pump equipped with a Shodex SUGAR KS804 column, using distilled water as the mobile phase (column temperature, 30 °C; flow rate, 0.4 ml min^{-1}). The molecular weight of MT-α-glucan was estimated based on a calibration curve made by HPGPC using Dextran T series glucan as standards. The relatively symmetric peak on HPGPC indicated the basically homogeneous polysaccharide fraction. The retention time was 11.775 min (Fig. 1). The molecular weight of MT-α-glucan was about 400,000–450,000 Da estimated by HPGPC.

Sugar composition was determined by thin-layer chromatography (TLC) analysis of the acid hydrolysed product and gas chromatography (GC) analysis of the acetylized product. The best acid hydrolysis condition was determined by the references (Liu et al., 2005; Zhang, 2002), and the previous studies on polysaccharides and initial studies on *G. frondosa* in our laboratory (Liu et al., 2005). Results of TLC analysis of the acid hydrolysed product and GC analysis of the acetylized product demonstrated that the polysaccharide was composed of D-glucose (Fig. 2), as the sole carbohydrate present in the polysaccharide chain. The primary structural features of the polysaccharide was characterized by Fourier transformed infrared (FTIR), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The main FTIR absorption bands were found at 3325, 2937, 1643, 1413, 1045, 923 and 858 cm^{-1} in the FTIR spectrum (Fig. 3). Among them, the bands at 923 and 858 cm^{-1} (C1–H deformation) are sensitive to anomeric configuration and indicated that this polysaccharide is alpha-glucan. ^{1}H and ^{13}C NMR spectra are shown in Figs. 4 and 5. The H-1 proton signal was found at delta 5.46 ppm, and the C-1 signal at delta 99.81 ppm. These signal positions also confirmed alpha-anomeric configuration of the isolated glucan. So the spectra obtained by FTIR, ^{1}H NMR and ^{13}C NMR analyses of the compound revealed that it contained an alpha-glucosidic bond. Thus, the main compound in the extract was demonstrated to be an alpha-glucan. The mode of linkage bond was analyzed by periodate oxidation, Smith degradation and methylation analysis, which indicated that there were four kind of linkage bond, that is T-, 1,4-, 1,3,6-, 1,4,6-glycosidic linkages, and the corresponding molar ratio is 5:29:1:3.

2.2. Main reagents

Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco. Streptozotocin (STZ) and MTT were from Sigma. Trypsin was from Invitrogen life Technologies. Various measuring kits were used during the study. These were as follows: insulin analysis kit (Shanghai Yuanxiang Medical Instrument Co., Ltd., Shanghai, China); superoxide dismutase (SOD), glutathione peroxidase (GSHpx), reduced glutathione (GSH) and malondialdehyde (MDA) measurement kit, nitric oxide (NO), NO synthase (NOS), inducible NO synthase (iNOS) measurement kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All the other biochemicals and chemicals used in the experiment were of analytical grade.

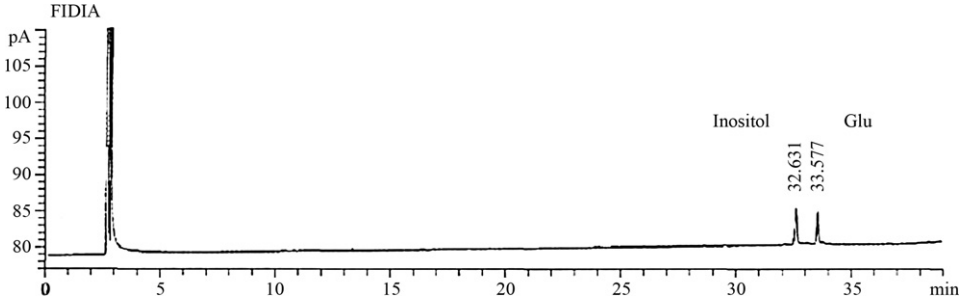

Fig. 2. GC spectrum of reduced alditol acetate of MT-α-glucan.

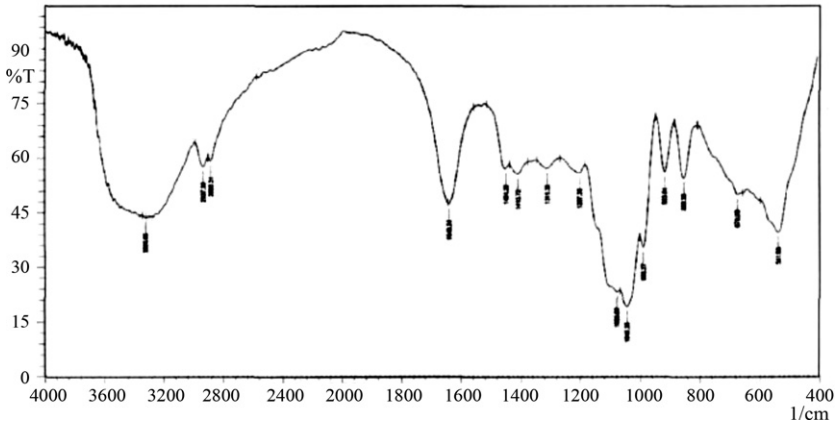

Fig. 3. FTIR spectrum of MT-α-glucan.

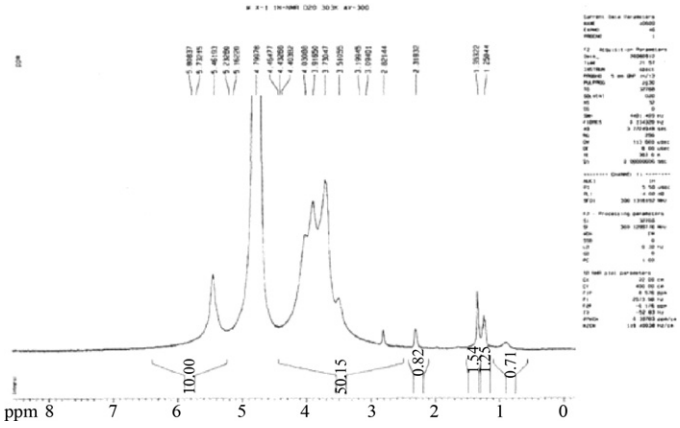

Fig. 4. ^{1}H NMR spectrum of MT-α-glucan.

2.3. NIT-1 cell culture

The pancreatic β-cell line NIT-1 was purchased from ATCC (NO. CRL-2055) and were cultured in Complete Dulbecco Minimum Essential Medium (DMEM) supplemented with 25 mM glucose, 15 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 2 g l^{-1} sodium bicarbonate, 100 mg l^{-1} penicillin/streptomycin, 10% heat-inactivated fetal calf serum (FCS) which were adjusted to pH 7.2, and maintained in 75 cm^2 tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2 incubators. Cells were allowed to attach to the flask. Cell culture medium was exchanged every 48 h and cells were passaged at weekly intervals by trypsination. Harvesting and passaging of the NIT-1 cells were accomplished by detaching, aspirating and separating the adherent cells by mechanical agitation, followed by incubation with 0.25% trypsin and 0.02% EDTA in D-Hank's solution (pH 7.2) for 1–2 min.

2.4. Cytotoxity of streptozotocin (STZ)

Cells were seeded onto 96 well plates at a cell concentration of 1×10^5 cells per well and were pre-incubated overnight. After pre-incubation, NIT-1 cells were exposed to toxin STZ. The cells were incubated for 24 h without or with different concentration of STZ 10 μl (dissolved in 0.02 M acetate buffer) and the final concentration was 0, 0.75, 1.5, 3, 6, 12 mM, respectively. Cytotoxicity of STZ on the NIT-1 cells was determined using MTT reduction assay.

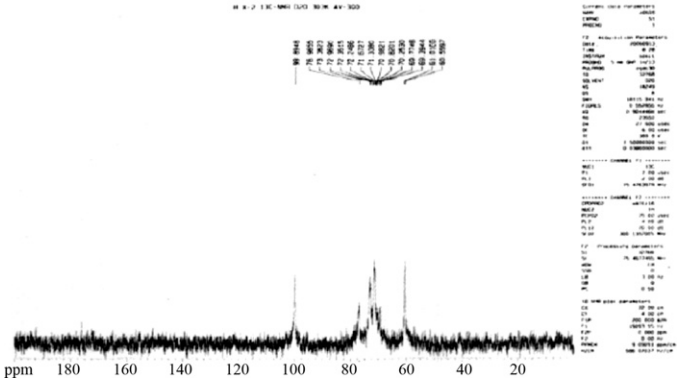

Fig. 5. ^{13}C NMR spectrum of MT-α-glucan.

2.5. MTT assay

Cells were seeded at 1×10^5 per well in a 96-well plate for viability assay. MTT solution (5 mg/ml in PBS) 20 μl was added to each well and the plates were further incubated for another 6 h. Supernatants were then discarded and DMSO 150 μl was added to the each incubation well and mixed thoroughly to dissolve the dark blue crystal formazan. The absorbance at 570 nm (formation of formazan) was recorded with a microplate spectrophotometer (Song et al., 2007).

2.6. Experimental design

Aliquots of 1×10^4 NIT-1 cells were transferred into the wells of 96-well cell culture plates. After 48 h, STZ solution (final concentration 6 mM) was added to each well of 96-well-plates and the cells were exposed to STZ for 24 h or were kept untreated as controls. Meanwhile, the cells were incubated for 24 h in the presence or absence of MT-α-glucan (dissolved in RPMI-1640 and the final concentration 400, 200, 100 μg ml^{-1}). The cell viability and condition were determined. Cell viability was determined by using a microtitre plate-based MTT assay, which mentioned above. Cell condition was evaluated under the reverted microscope. Biochemical measurements of the supernatant and the harvested cells were also evaluated.

2.7. Microscopic observation

Visual observations of NIT-1 cells under the inverted microscope were evaluated. The appearance of NIT-1 cells were observed under light microscope to evaluate the cell shape, the integrity of the cell membranes, confluence of the monolayer and the portion of dead cells.

2.8. Biochemical measurements

The supernatants and the harvested cells in each well were collected and were used for biochemical assay. Estimation of levels of insulin secreted into the medium was assayed. The antioxidative capacity such as the activity of SOD and GSHpx, the content of GSH and MDA of NIT-1 cells were evaluated. The content of NO, the activity of NOS and iNOS of NIT-1 cells were also assayed. The above biochemical parameters were determined by using commercial kits according to the guidelines indicated. All samples were assayed in triplicate.

2.9. Statistical analysis

Data were expressed as means ± s.d. Statistical analysis was evaluated by one-way analysis of variance, followed by the Student–Newman–Keuls test for multiple comparisons, which was used to evaluate the difference between two groups. $P < 0.05$ was considered significant.

3. Results

3.1. Cytotoxicity of streptozotocin (STZ) on the pancreatic NIT-1 cells

NIT-1 β cells were MTT assayed for viability which was expressed as the value of O.D. at 570 nm. The different concentration of STZ on the viability of NIT-1 cells was shown in Fig. 6. In vitro, administration of STZ to the NIT-1 cells for 24 h caused dose-dependent toxicity. Viability of NIT-1 cells reduced to a greater extent. STZ had a significant adverse effect on cells from the concentration of 1.5 mM compared with the untreated group ($P < 0.01$).

Table 1
Effect of MT-α-glucan on cellular SOD, GSHpx, GSH and MDA in NIT-1 cells.

Group	Dose($\mu g\,ml^{-1}$)	SOD ($U\,mg^{-1}$ protein)	GSHpx ($U\,mg^{-1}$ protein)	GSH ($mg\,g^{-1}$ protein)	MDA ($nmol\,mg^{-1}$ protein)
Normal control	–	15.32 ± 2.77*	14.22 ± 2.26*	36.46 ± 5.23*	0.483 ± 0.119*
STZ model	–	11.67 ± 1.60	11.29 ± 1.21	28.09 ± 4.35	0.739 ± 0.138
MT-α-glucan	100	13.88 ± 2.42	13.16 ± 1.87	32.99 ± 3.73	0.570 ± 0.130
	200	14.98 ± 2.05*	13.33 ± 1.79	36.78 ± 4.16*	0.456 ± 0.062**
	400	16.10 ± 2.08**	14.35 ± 1.64*	38.14 ± 4.62*	0.471 ± 0.068**

Data are the mean ± s.d. ($n = 10$). Analysis of variance followed by the Student–Newman–Keuls test. In each vertical column,
* $P < 0.05$ compared with STZ model control group.
** $P < 0.01$ compared with STZ model control group.

At 6 mM of STZ, nearly 50% of the cells were dead and at the concentration of 12 mM, most of the cells (80%) were dead. We selected 6 mM of STZ to do the further experiment.

3.2. Effect of MT-α-glucan on the viability of NIT-1 cells damaged by STZ

We investigated the protective effect of MT-α-glucan on the damaged NIT-1 cells induced by STZ toxicity. As shown in Fig. 7, cell viability significantly decreased in STZ-model control group compared with the normal control ($P < 0.01$). Administration of MT-α-glucan (400, 200 $\mu g\,ml^{-1}$) for 24 h significantly reversed STZ-induced cells viability loss, the O.D. values significantly increased ($P < 0.05$ or 0.01). This indicated that treatment with MT-α-glucan significantly protected the NIT-1 cells from STZ-induced cell death.

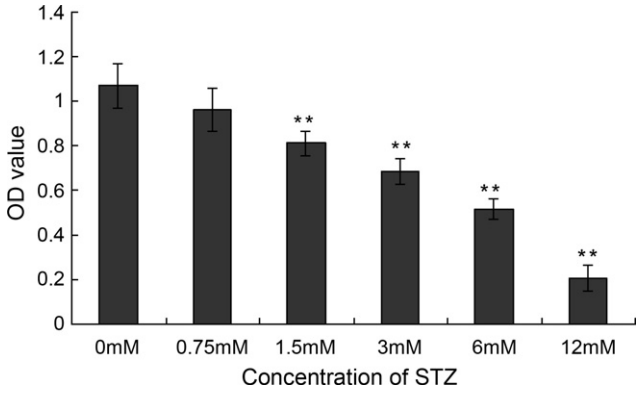

Fig. 6. Effect of different concentration of STZ on the NIT-1 cell viability. Data are the mean ± s.d. ($n = 10$). Analysis of variance followed by the Student–Newman–Keuls test. In each vertical column, *$P < 0.05$ and **$P < 0.01$ compared with 0 mM control group.

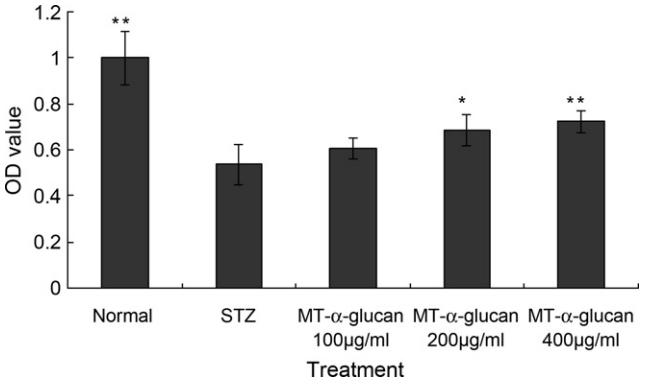

Fig. 7. Effect of MT-α-glucan on the NIT-1 cell viability damaged by STZ. Data are the mean ± s.d. ($n = 10$). Analysis of variance followed by the Student–Newman–Keuls test. In each vertical column, *$P < 0.05$ and **$P < 0.01$ compared with STZ model control group.

3.3. Effect of MT-α-glucan on insulin secretion by NIT-1 cells damaged by STZ

As shown in Fig. 8, STZ had an adverse effect on insulin secretion by NIT-1 cells. Insulin production of STZ-treated cells decreased significantly compared with the normal control ($P < 0.01$). MT-α-glucan (400, 200 $\mu g\,ml^{-1}$) exerted significant promoting actions on insulin secretion by NIT-1 β-cell compared with the STZ model control ($P < 0.05$). This suggested that MT-α-glucan has the improving effect on insulin secretive function of NIT-1 cells, which may be the consequence of protection of the cells damaged by STZ.

3.4. Effect of MT-α-glucan on levels of SOD, GSHpx, GSH and MDA in NIT-1 cells

The changes of the levels of SOD, GSHpx, GSH and MDA of normal, model and MT-α-glucan groups were shown in Table 1. Results showed that the activity of T-SOD, GSHpx and the content of GSH of STZ-treated NIT-1 cellular model markedly decreased compared with the normal control ($P < 0.05$), while the content of MDA markedly increased compared with the normal control ($P < 0.05$). T-SOD, GSHpx activity and GSH content markedly increased, MDA content markedly decreased in MT-α-glucan (400, 200 $\mu g\,ml^{-1}$) group compared with the normal control group ($P < 0.05$ or 0.01). This suggested that MT-α-glucan has anti-oxidative effect, which prevents and protects STZ-induced oxidative stress and β-cell damage.

3.5. Effect of MT-α-glucan on levels of nitric oxide (NO), NOS, iNOS in NIT-1 cells

The levels of NO, NOS and iNOS were shown in Table 2. The levels of NO, NOS and iNOS were significantly higher in STZ-treated NIT-1 cells as compared with normal control ($P < 0.05$ or $P < 0.01$).

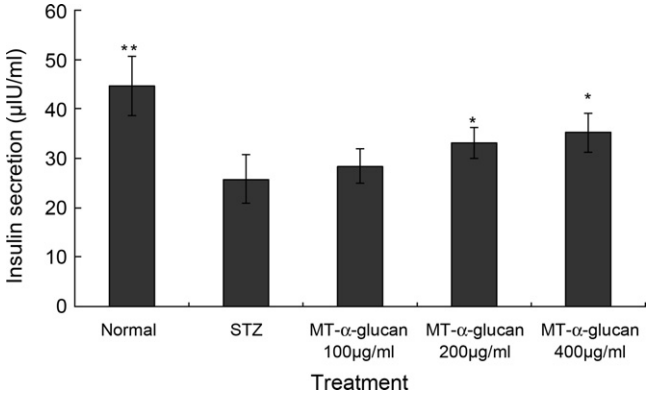

Fig. 8. Effect of MT-α-glucan on insulin secretion by NIT-1 cells damaged by STZ. Data are the mean ± s.d. ($n = 10$). Analysis of variance followed by the Student–Newman–Keuls test. In each vertical column, *$P < 0.05$ and **$P < 0.01$ compared with STZ model control group.

Table 2
Effect of MT-α-glucan on cellular NO production by NIT-1 cells.

Group	Dose (μg ml^{-1})	NO (μmol l^{-1})	NOS (U ml^{-1})	iNOS (U ml^{-1})
Normal control	–	61.81 ± 5.57*	2.04 ± 0.20**	0.65 ± 0.22*
STZ model	–	85.70 ± 18.36	2.86 ± 0.45	1.04 ± 0.27
MT-α-glucan	100	72.47 ± 3.59	2.39 ± 0.29	0.89 ± 0.23
	200	62.89 ± 7.75*	2.13 ± 0.21*	0.82 ± 0.23
	400	61.47 ± 5.17*	2.14 ± 0.21*	0.68 ± 0.17*

Data are the mean ± s.d. (n = 10). Analysis of variance followed by the Student–Newman–Keuls test. In each vertical column,
* $P < 0.05$ compared with STZ model control group.
** $P < 0.01$ compared with STZ model control group.

STZ might lead to the destruction of the pancreatic β cells by induction of increased expression of NOS and iNOS. Incubation of NIT-1 cells with MT-α-glucan (400, 200 μg ml^{-1}) lowered the NO, NOS, iNOS levels markedly as compared with untreated model ($P < 0.05$). This suggested that MT-α-glucan has the effect of suppressing the generation of NO, which is an another β-cell destructive factor, and exerts β-cell-protecting effect.

3.6. Effect of MT-α-glucan on the microscopic observation of NIT-1 cells damaged by STZ

Healthy NIT-1 cells were observed under microscope (Fig. 9A). The shape of normal NIT-1 cells was irregular polygon, adherent to the plate-wall in cluster aggregation. When incubation with STZ, the number of NIT-1 cells markedly decreased, and the shape markedly pathological changed such as cell shrinkage, dark appearance and the black-spot could been seen under microscope. Moreover, STZ also had a greater effect in reducing confluence of the NIT-1 cells (Fig. 9B). This suggested that the damaged β-cellular model induced by STZ was successfully established. Treatment with MT-α-glucan (400, 200 μg ml^{-1}) markedly restored the shape and structural integrity of the damaged cells as compared with model control (Fig. 9C and D). This indicated that the pancreatic NIT-1 β-cells were obviously destroyed by STZ, while MT-α-glucan could ameliorate NIT-1 β-cell-destruction significantly and had β-cell-protecting effect.

4. Discussion

The procedure of preparation of MT-α-glucan was studied in our laboratory. The purity of the compound estimated by HPGPC demonstrated that the molecule was basically homogeneous, and the molecular weight was about 400,000–450,000 Da. Results of spectroscopic analyses (FTIR, ^{1}H NMR, ^{13}C NMR) and monosaccharide composition analyses (TLC, GC) demonstrated that the molecule is an alpha-glucan rather than a beta-glucan, hitherto

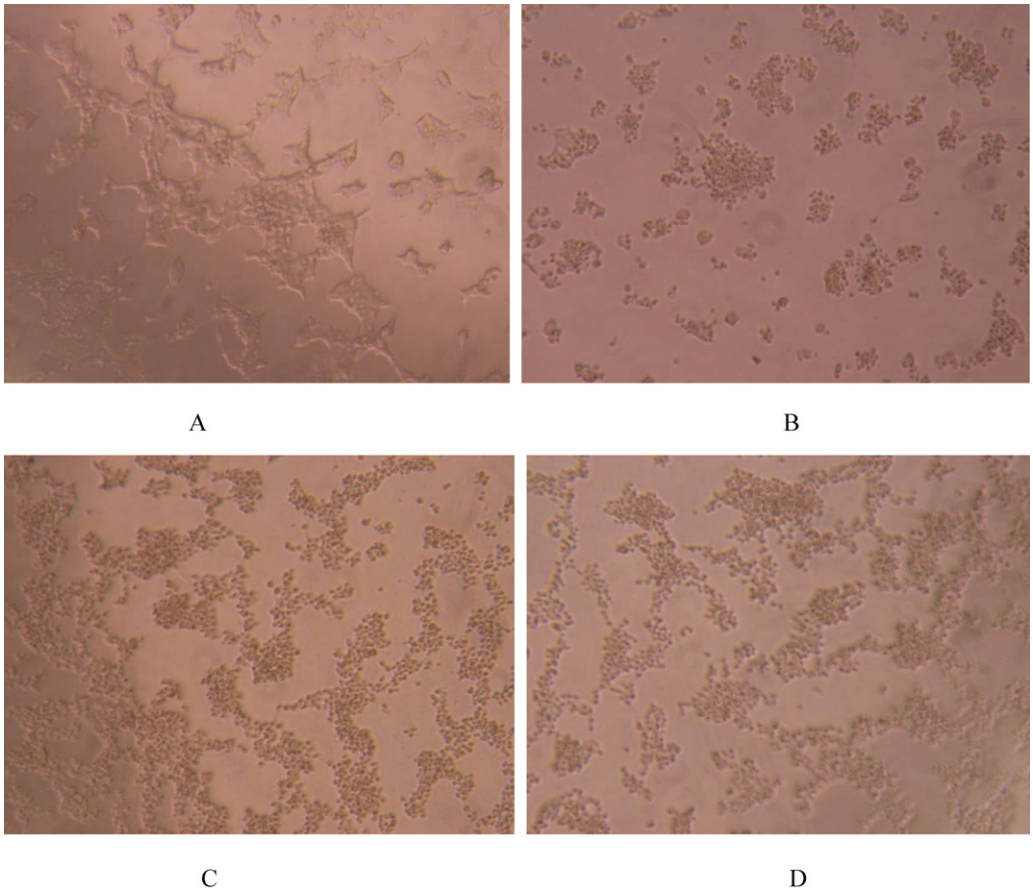

Fig. 9. Effect of MT-α-glucan on microscopic observation of NIT-1 cells. (A) Normal control group; (B) STZ model group; (C) MT-α-glucan 400 μg ml^{-1} group; (D) MT-α-glucan 200 μg ml^{-1} group.

reported to be most commonly produced by this mushroom strain (Kubo, 1994; Kubo & Nanba, 1998). So this molecule is unique to maitake among mushrooms according to references we have searched. Initial studies on primary structure of MT-α-glucan showed that the glycosidic linkage bond is mainly alpha-1,4-glycosidic linkages, containing a few 1,3,6-,1,4,6-linkage bonds. Further studies on primary structure of MT-α-glucan such as partial hydrolysis with acid are under investigation in our laboratory. The biological activity of MT-α-glucan may be related to the different kind of alpha glycosidic linkages. The mechanism of action of the relationship between polysaccharide structure and properties will be studied in the following research.

The NIT-1 cell line was established from the insulinomas that developed in the transgenic NOD mice, and the cells were transformed with a hybrid rat insulin promoter/SV40 large T-antigen. Preliminary work on the NIT-1 cell line showed that it possessed many characteristics and ultrastructural features of normal differentiated mouse pancreatic β cells, such as well developed rough endoplasmic reticulum, extensive golgi apparatus and beta granules (Hamaguchi, Gaskins, & Leiter, 1991). NIT-1 cells provide a substantial supply of immortalized β cells, which are normally difficult to obtain.

Streptozotocin (STZ), an antibiotic produced by *Streptomyces achromogenes*, has the β-cell cytotoxic effect and is a kind of common used agent in experimental diabetes. In STZ induced diabetes, hyperglycemia and β-cell destruction have been implicated in the etiology and pathology of diabetes (Luo et al., 1998). Although the mechanism of β-cell cytotoxic action of STZ is not fully established, it is thought to act through oxidative damage, mediated by the inhibition of free radical scavenger-enzymes and thereby enhancing the production of the superoxide radical and NO. Chemicals with antioxidant properties and free radical scavengers were shown to prevent pancreatic islets against cytotoxic effects of STZ (Jia, Xin, Hu, Ren, & Wang, 2000).

We have recently demonstrated that treatment with MT-α-glucan (300, 100 mg kg^{-1}) attenuated the degree of injured β-cells of pancreatic islets of type 2 diabetes experimental mice induced by high-fat diets and STZ in vivo (Lei et al., 2012). In the present study, we adopted STZ-induced NIT-1 cell-injury model to investigate the protective effect of MT-α-glucan on β cells in vitro. Results of the present study showed that NIT-1 cells incubated with STZ for 24 h suffered concentration-dependent decrease in cell viability measured by MTT assay. Incubation with MT-α-glucan for 24 h protected NIT-1 cells from being damaged by STZ toxicity. NIT-1 cells showed significantly higher viabilities co-incubation with MT-α-glucan. Microscopic observation results also showed that the protective effect of MT-α-glucan on cellular integrity and apparent shape destroyed by STZ. MT-α-glucan treatment in vitro also improved insulin secretion by NIT-1 β cells, suggesting that MT-α-glucan could directly improve the function of pancreatic β cells.

In vitro and in vivo studies have suggested the implication of oxidative stress in the progression of β-cell dysfunction in type 2 diabetes (Zhang, He, Yuan, & Lin, 2003). It has been generally accepted that pancreas is especially susceptible to STZ-induced free radical damage and low levels of key enzymes scavenging oxygen free radicals. Recently, we reported that MT-α-glucan has the anti-oxidative effect on KKay mice, a kind of type 2 diabetes animal model (Lei et al., 2007). In the present study, we investigate the effects of MT-α-glucan on the antioxidative activity of NIT-1 cells damaged by STZ in vitro. In order to determine the changes of cellular antioxidant defense system, antioxidant enzymes such as GSH_{Px}, SOD activities and antioxidative molecule GSH content were measured. Results showed that STZ induced the increased lipid per-oxidation and the decreased antioxidant enzyme activity significantly. Treatment with MT-α-glucan could reduce the content of the lipid per-oxidation product MDA in the cells, and increase the activity of the anti-oxidative defense system and thus inhibit free radical generation.

It has been known that NO is involved in the pathogenesis of diabetes and the functional impairment of islet β cells and insulin production (Hirotada, Noriko, & Hiroaki, 2000). In addition to the macrophages which may be the major source of NO production in the process of islet inflammation, β-cells themselves have been shown to induce NOS and generate NO (Ji, Ji, & Won, 2006). We have recently reported that administration of MT-α-glucan to T2DM mice could inhibit NO production by macrophages in vivo (Lei et al., 2012). In the present study we investigated whether MT-α-glucan may protect NIT-1 β-cells from the destructive actions of NO induced by STZ in vitro. Our results demonstrated that MT-α-glucan prevented the generation of NO by NIT-1 cells induced by STZ, and could inhibit the NOS, iNOS activity of pancreatic β-cells in vitro. Taken together these data suggests that MT-α-glucan mediates protection of β-cells not only from the cellular oxygen radical defense system but also from attenuation of NO toxicity.

5. Conclusion

Our study confirms that MT-α-glucan could protect pancreatic β cells damaged by streptozotocin (STZ) in vitro. The mechanisms may be related to its promoting cellular defense system by decreasing lipid per-oxidation and NO toxicity, increasing antioxidant enzyme activity, and consequently, preserving the integrity and function of pancreatic β cells.

Acknowledgment

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