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ANTI-DIABETIC EFFECTS OF GLUCOMANNO-OLIGOSACCHARIDES VIA AMPK ACTIVATION IN C2C12 MYOTUBES

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Abstract. In this paper, antidiabetic activities of glucomanno-oligosaccharides (GMO) were *in vitro* and *in vivo* investigated. Treatment with 100 μg/ml and 50 μg/ml of GMO for 1 hour caused 1.47-fold and 1.81-fold phosphorylation of AMPK, respectively. Oral administration of GMO (6 g/kg^{-1} of body weight day^{-1}) lowered blood glucose levels ($p < 0.05$) at 120 min as compared to control group. These results suggested that GMO exhibited anti-diabetic effects via activation of AMPK and could be useful for diabetes prevention.

Keywords: glucomanno-oligosaccharides, glucose uptake, AMP-activated protein kinase, C2C12 myotube.

Classification numbers: 1.2.1, 1.4.2, 1.4.8.

1. INTRODUCTION

Glucomannan is a neutral polysaccharide extracted from the tuber of *Amorphophalluskonjac* K. Koch. Konjacglucomannan consists of D-mannose and D-glucose units at a molar ratio of about 1.6:1.0. These units are joined by β-(1,4)-glycosidic bonds to form the backbone, and a few branches are formed by β-(1,6)-glycosidic units. These sugar units with acetyl groups comprise about 1/9th to 1/20th of the total sugar units [1]. The molecular weight of native konjacglucomannan is between 10^5 - 10^6 Da, depending on species and habitat [2].

Glucomannan, a soluble fiber, improves satiety by providing bulk and increasing digestion time to slow postprandial glucose uptake, thereby producing lower blood glucose and insulin levels [3]. Glucomannan cannot be hydrolyzed by digestive enzymes in human upper gastrointestinal tract and is therefore regarded as an indigestible dietary fiber which has also been demonstrated to be effective in weight reduction, regulation of lipid metabolism and cholesterol reduction [4]. β-glycosidic linkages between the glucose and mannose building blocks (β-(1→4) linkages in the main chain and β-(1→3) linkages at the branch points) make

konjacglucomannan a non-digestible polysaccharide [3]. Despite its hydrophilicity, glucomannan is poorly soluble in water (solubility of around 30 %) due to its high molecular weight, which limits its application range in certain areas. Recent research found that its degradation products with different molecular weights have particular biological functions, such as antitumor [5], immune regulation [6], cytotoxicity [7], antioxidant activity [4]. Glucomanno-oligosaccharides also have shown better probiotic functions than its parent unhydrolyzedglucomannan [8]. According to Min Jiang *et al*., glucomanno-oligosaccharides could be a candidate for use as a tool for the treatment of various diseases, including intestinal flora imbalance, and oxidative- and immune-related disorders [9]. In addition, it could also reduce the risk of gut cancer, restrict aflatoxin toxicity, and used as drug carriers with designed pharmacokinetic behaviors [10].

Diabetes mellitus ranks highly with the top ten disorders which causes of death. It is not a single disease entity but a set of metabolic disorders with a common underlying feature of high blood glucose level. Skeletal muscle is the primary peripheral tissue responsible for blood glucose control, and more than 70 % of insulin- mediated glucose uptake occurs here [11]. The AMP-activated protein kinase (AMPK) is an enzyme that is activated in situations where there are changes in the cellular energy status such as muscle contraction and hypoxia [12, 13]. AMPK can also be pharmacologically activated by the compound 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and the antidiabetic agent metformin. Several studies support the hypothesis that AMPK plays an important role in the stimulation of muscle glucose uptake by these physiological and pharmacological stimuli. The effects of AMPK on muscle glucose uptake makes this protein a promising pharmacological target for the treatment of type 2 diabetes [14].

The objective of this research was to evaluate the anti-diabetic effect of GMO via AMPK activation in C2C12 skeletal muscle cells. The ability to stimulate blood glucose tolerance of GMO was determined in normal, healthy mice by oral glucose tolerance test (OGTT). To the best of our knowledge, antidiabetic potential of GMO has rarely been reported before. This is also the first report of *in vivo* and *in vitro* anti-diabetic activities of GMO in Viet Nam.

2. MATERIALS AND METHODS

2.1 Materials

GMO (Mw ≅ 2050 Da) was prepared by endo-1,4 β-mannanase from bacteria *Bacillus* sp. in our Lab as described before [15, 16].

The C2C12 myoblasts (CRL-1772) were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). Male C57BL/6J mice were obtained at 5 weeks of age from Vietnam National Institute of Hygiene and Epidemiology. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), horse serum (HS), and penicillin−streptomycin (PS) were obtained from WelGENE (Daegu, Korea). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. AMPK activation by GMO in vitro

The C2C12 myoblasts were cultured in DMEM containing 10 % FBS and 1 % PS at 37 °C in humidified air containing 5 % CO_2. When the myoblasts were confluent, they were treated with differentiation inducers (DMEM containing 1 % HS) for 5 days. The experiments were

performed in differentiated C2C12 myotubes. C2C12 myotubes were incubated with DMSO or different concentrations of GMO: 6.25; 12.5; 25; 50; 100 μg/mL, labeled as GMO1, GMO2, GMO3, GMO4 and GMO5.

After experiments were completed, cells were washed twice with ice-cold PBS, and lysed with lysis buffer (50 mMTrisHCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 150 mM sodium chloride (NaCl), 1 mMethylenediaminetetraacetic acid (EDTA), 1 mMphenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 1 mMNaF and 0.2 % protease inhibitor cocktail; pH 7.2.

The protein concentration of cell lysates was determined using the Bradford assay, and an equal quantity of protein was separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (10 %). The protein was transferred to a polyvinylidene fluoride membrane at 0.2 A for 90 min, and the membrane was blocked with 5 % bovine serum albumin (BSA) in Tris-buffered saline and 0.1 % Tween 20 (TBS-T) for 50 min at room temperature. The membrane was incubated for two days at 4 °C with the primary antibody solution. The membrane was incubated with the horseradish peroxidase (HRP)-labeled HRP- conjugated secondary antibody and rinsed again. HRP catalyzes the oxidation of luminol in the presence of hydrogen peroxide. The image of the blot is visualized by exposing the blot to film. ImageJ software was used to determine the emission of light obtained on films. Compare the light ratio of protein strips to β-actin between the control group and the sample incubation groups to evaluate the effect of glucomannan on the expression of these proteins [17]. All quantitative determinations were carried out in triplicate and data were statistically processed according to Microsoft excel software. The difference was statistically significant if $p < 0.05$.

2.2.2. Oral Glucose Tolerance Test (OGTT)

The animals were maintained at 22 ± 1 °C and 50 ± 10 % relative humidity, with a 12 h light (from 7 am to 7 pm)/dark cycle. The mice were fed daily with synthetic feed supplied by the Institute of Vaccines and Biologicals. After 7 days of acclimatization, animals (21.9 ± 1.8 g) were randomly assigned to six groups. The mice were divided into test groups, each with 10 individuals. Group 1 served as control, the animals were fed with distilled water only. Group No2, 3, 4 received GMO at a dose of 3 g/kg, 6 g/kg, and 9 g/kg body weight per day (B.W.day^{-1}), respectively. Group 5 received gliclazide at a dose of 10 mg/kg body weight. Glucose solution (2 g/kg, B.W day^{-1}.) was administered 30 min after drug administration. Blood samples were collected at the 0, 30, 90 and 150 min after glucose load.

3. RESULTS AND DISCUSSION

3.1. AMPK activation by GMO in vitro

AMPK is known as a metabolic sensor. It up-regulates catabolic pathways that generate ATP and down-regulates anabolic pathways that consume ATP. AMPK is recognized as one of the crucial targets for the prevention and treatment of obesity and type 2 diabetes [18]. The relative phosphorylation levels of AMPK-Thr172 (p-AMPK) in C2C12 myotubes was shown in Fig.1 and the phosphorylation/total AMPKα ratio in skeletal muscle compared to that in the control group was shown in Fig. 2. GMO significantly increased the phosphorylation of AMPKα at Thr172 (the results represent the mean ± SD performed in triplicate). GMO increased AMPK phosphorylations in a dose dependent manner. Treatment with GMO 100 μg/ml and 50 μg/ml

caused 1.47-fold and 1.81-fold phosphorylation of AMPK, respectively. The elevated glucose uptake in C2C12 myotubes induced by GMO may be mediated by GLUT4 translocation. However, further studies are warranted to examine how AMPK is activated by GMO.

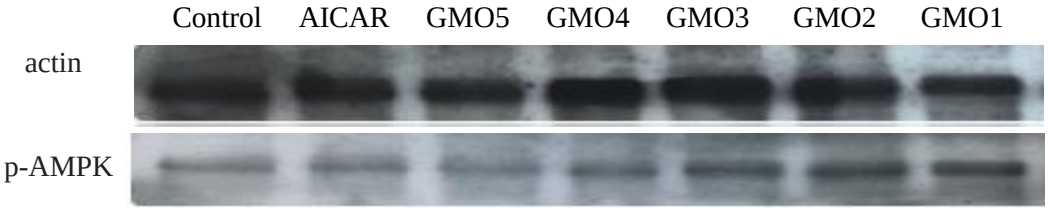

Figure 1. The relative phosphorylation levels of AMPK-Thr172 (p-AMPK) in C2C12 myotubes.

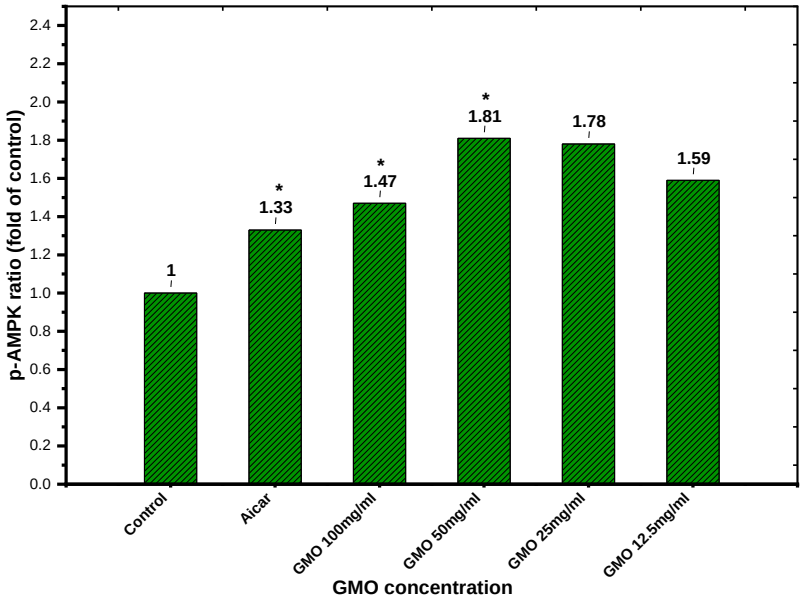

Figure 2. Phosphor-AMPK (Thr172)/total AMPK ratio.

3.2. Oral glucose tolerance test (OGTT) *in vivo*

In Viet Nam, the tuber of *Amorphophallus* sp.– Araceae family has been used as a food source (especially in Vietnamese Famine of 1945) and as a traditional Vietnamese medicine for a long time, so glucomannan and GMO are none-toxic (data not shown).

The management of postprandial blood glucose levels is important in practical terms. For Oral Glucose Tolerance Test, the blood samples were analyzed for glucose content at -30, 0, 30, 60, 120 minutes, respectively. The results were shown in the Table 1. Blood glucose levels had rapidly increased 30 min after administration of the glucose load. The doses at 3 g/kg produced no significant hypoglycemic effect in normal mice ($p > 0.05$). However, GMO at the dose of 6 g/kg significantly attenuated the elevated blood glucose levels seen following glucose loading at the time of 30 and 60 min ($p < 0.05$). It demonstrated noteworthy anti-hyperglycemic effect from 60 min onward ($p < 0.05$). The results indicated that GMO treatment increased utilization of peripheral glucose in mice, resulting in improved glucose tolerance. GMO showed concentration-dependent reduction in the blood glucose level. The results were compared with

gliclazide that has been used for many years to treat diabetes and stimulated insulin secretion. This study provides good evidence that GMO has potential to be used as a functional food for glycemic control.

Table 1. The end of oral glucose tolerance test (OGTT) result.

Treatment	Blood glucose levels (mg/dl)				
	-30	0	30	60	120
Control	4.51 ± 0.51	3.98 ± 0.44	5.32 ± 0.14	5.02 ± 0.25	4.12 ± 0.25
Group 2 (GMO3 g/kg b.w)	4.42 ± 0.51	3.53 ± 0.34	5.58 ± 0.24	5.17 ± 0,31	4.04 ± 0.41
Group 3 (GMO6 g/kg b.w)	4.28 ± 0.59	3.22 ± 0.43	5.35 ± 0.55^{a}	5.05 ± 0.43^{a}	4.18 ± 0.55
Group 4 (GM6 g/kg b.w)	4.38 ± 0.28	3.58 ± 1.14	5.55 ± 0.32	5.12 ± 0.23^{a}	4.10 ± 0.23
Gliclazide (10 mg/kg)	4.4 ± 0.62^{b}	3.17 ± 0.51^{b}	4.06 ± 0.11^{b}	3.74 ± 0.29^{b}	3.17 ± 0.66^{b}

Superscript letters a: $p < 0.05$, superscript letters b: $p < 0.01$.

4. CONCLUSIONS

In conclusion, the present study demonstrates that GMO exhibited anti-diabetic effects via activation of AMPK in a concentration-dependent manner. At the dose of 6 g/kg, GMO significantly attenuated the elevated blood glucose levels ($p < 0.05$). Our findings provide a scientific basis for the use of GMO for the prevention and treatment of diabetes.

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