Sorbitol has no significant effects on 3T3-L1 -adipogenesis and adiponectin synthesis and secretion

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Abstract

Sorbitol is a sugar alcohol, widely used as a low-calorie sweetener. It has been shown that sorbitol has no effects on blood glucose. Decreased plasma adiponectin, secreted by adipocytes in adipose tissues, is associated with insulin resistance, type-2 diabetes and cardiovascular diseases. The research objectives were to determine if sorbitol would affect adipogenesis of pre-adipocytes and adiponectin production from mature adipocytes. Adipogenesis was assessed by measuring fat accumulations in mature adipocytes by using Oil Red O staining. Adiponectin production was determined by using SDS-polyacrylaminde gel electrophoresis and Western blot technique. Pre-adipocyte 3T3-L1 cells were used as a model of study. First, adipogenesis of pre-adipocyte 3T3-L1 cells was induced in the absence and presence of sorbitol. It was found that adipogenesis was not significantly changed by sorbitol. Next, mature 3T3-L1 adipocytes were treated with sorbitol for 24 and 48 hours, and adiponectin in the adipocyte lysates and the culture media was determined to assess adiponectin synthesis and secretion. It was observed that sorbitol did not affect adiponectin synthesis and secretion from the mature 3T3-L1 adipocytes. Our results reveal that sorbitol does not directly affect 3T3-L1 cell adipogenesis and adiponectin production.

Keywords: 3T3-L1 cells; adipocyte; adipogenesis; adiponectin; sorbitol; sweetener.

1. Introduction

Sorbitol, also known as glucitol, is a sugar alcohol, found naturally in fruits, including apples, pears and cherries. Sorbitol is approximately 60% as sweet as sucrose [1]. It is therefore added as a low-calorie sweetener to several kinds of food products such as diet food, sugar-free chewing gum, and medications [2-4]. In addition, it is used in insulin-dependent diabetics because it does not affect blood glucose [5]. Sorbitol is slowly absorbed in the intestines though passive diffusion, enters cells via the triose part of the glycolytic pathway, and is mainly metabolized in the liver [6, 7]. However, a sorbitol intake of more than 30-50 g/day may cause osmotic diarrhea [8]. Sorbitol is oxidized to fructose by sorbitol dehydrogenase and enter the same metabolic pathway as fructose [9]. It has been shown that fructose significantly induces lipogenesis, leading to increased hepatic fatty acids, which can be either deposited as ectopic liver fat (hepatic steatosis) or secreted as VLDL-triacylglycerols [10, 11]. In additions, high fructose intake causes insulin resistance and increases the risks of diabetes and cardiovascular diseases [12]. Furthermore,

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fructose can induce adiponectin gene expression in 3T3-L1 adipocytes [13]. Adiponectin is a peptide hormone which is synthesized as a monomer by adipocytes in adipose tissue. The monomer polymerizes to polymers, which can be detected as trimers, hexamers and oligomers [14]. Oligomeric forms of adiponectin are biologically active and are reduced in diabetics [15]. Plasma levels of adiponectin are also reduced in subjects with obesity, insulin resistance, type-2 diabetes and cardiovascular diseases [16-18]. Furthermore, adiponectin helps reduce fat accumulation in macrophages and foam cells during atherogenesis by suppressing oxidized LDL uptake and increasing HDL-mediated cholesterol efflux [19, 20]. Adiponectin binds its receptor in skeletal muscle and liver and stimulates the adenosine monophosphate-activated protein kinase pathway [21], leading to increases in β-oxidation of fatty acids and insulin sensitivity by translocation of GLUT4 to plasma membrane. Consequently, hepatic gluconeogenesis and glucose output are reduced [22].

As mentioned earlier, sorbitol shares the same metabolic pathway as fructose, which induces lipogenesis and adiponectin expression and also increases the risks of diabetes and cardiovascular diseases. Therefore, we hypothesized that sorbitol would have similar effects to fructose specifically that it would affect adipogenesis and adiponectin gene expression. The objectives of the present study were to determine effects of sorbitol on lipogenesis by assessing adipogenesis of pre-adipocyte 3T3-L1 cells and on adiponectin synthesis and secretion by 3T3-L1 mature adipocytes.

2. Methods and Materials
2.1 Chemical reagents

Mouse 3T3-L1 pre-adipocytes (American type culture collection, Manessas, VA) were kindly provided by Associate Professor Sarawut Jitrapakdee (Biochemistry department, faculty of Sciences, Mahidol University, Thailand). Dulbecco modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Gibthai, Thailand). Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), Oil Red O, sorbitol, insulin, cycloheximide, Troglitazone and GW9662 were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Antibodies used included mouse anti-adiponectin (Chemicon International, Inc., Temecula, CA), mouse anti-β-actin (Santa Cruz Biotechnology, California, USA), mouse anti-PPARγ (E-8; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and goat anti-mouse HRP conjugates (Bio-Rad Laboratories, Hercules, CA).

2.2 Cell cytotoxicity

MTT (Thiazolyl Blue Tetrazolium Bromide) assay [23, 24] was used to study cell viability after sorbitol treatment. MTT assay is based on the principle that viable cells contain enzymes that can metabolize the MTT tetrazolium dye to purple insoluble formazan product, reflecting cell viability quantities. Pre-adipocyte 3T3-L1 cells (about 10,000 cells) were cultured in 96-well plates at $37^{o}C$ in 5% CO_2. After cell attachment, pre-adipocyte 3T3-L1 cells were further incubated with PBS (control) or sorbitol (5, 10, 50, and 100 mM) for 48 hours. After that, cell viability was assessed by fresh MTT solution (20 μL of 5 mg/mL), followed by incubation at $37^{o}C$ for 4 hours to allow the MTT to be metabolized, solubilization of formazan product with 200 μL of dimethyl sulfoxide (DMSO), and measurement of the optical density (OD) at 570 nm against background wavelength at 630 nm. The percentages of viable cells (percent) were then calculated to control (cells treated with PBS), using the following formula: viable cells (%) = [OD value of sample / OD value of control] x 100.

2.3   Adipogenesis assay by Oil Red O staining

Differentiation or adipogenesis of 3T3-L1 was studied using the adipogenesis assay kit (Chemicon international, Inc., Temecula, CA). Six thousands per well of 3T3-L1 cells were cultured in 24-well plates in 10% FBS-containing DMEM only (negative control) or supplemented with adipogenesis inducer (IBMX and dexamethasone) alone (adipogenesis control) or with the inducer and additional treatments [sorbitol (0.5, 5, and 50 mM), adipogenesis activator (10 μM PPARγ agonist; troglitazone) or inhibitor (5 μM PPARγ antagonist; GW9662)] for 2 days. Then, IBMX and dexamethasone were substituted with insulin and cells were further incubated for 2 days. After that, cells were further incubated in culture media without insulin supplement for 3 days. After seven days of differentiation, cells were stained with Oil Red O. Then, the dye was extracted, and the extracted dye was transferred to 96-well plates. Finally, the absorbance of extracted Oil Red O was measured at 490 nm.

2.4   Adiponectin and PPARγ analysis

Pre-adipocyte 3T3-L1 cells were cultured in DMEM containing 25 mM glucose (DMEM/High glucose), supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO_2. To induce adipogenesis, cells were grown to confluence in 6-well plates and stimulated after two days of post-confluence with the adipogenesis induction medium (0.5 mM IBMX, 250 nM dexamethasone, and 5 μg/mL insulin in 10% FBS-containing DMEM) for 48 hours. Then, cells were cultured in 3T3-L1 adipocyte medium (DMEM containing 10% FBS and 5 μg/mL insulin). The 3T3-L1 adipocyte medium was changed every two days. When 3T3-L1 adipocytes attained maturity, which is about 6 days after the induction, the culture medium was changed to a serum-free medium (DMEM/low glucose) with and without sorbitol treatments. After that, cells were further incubated for 24 or 48 hours and then treated with 10 μg/mL cycloheximide (CHX) for 8 hours [25] to stop new protein synthesis. The media and cells were then harvested and prepared for analysis of the adiponectin and PPARγ.

2.5 SDS-PAGE and Western blot

Cell lysates or culture media were incubated with the sample buffer (20 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 16% (v/v) glycerol, and 0.004% (w/v) bromophenol blue) containing 10% dithiothreitol (DTT), as a reducing agent, for 5 min at 100°C, to change oligomeric to monomeric forms, and resolved in 12.5% (w/v) SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to nitrocellulose membranes. After that, the membranes were incubated overnight with mouse anti-adiponectin antibody (1:5,000), mouse anti-mouse PPARγ antibody (1:100) or mouse anti-actin antibody (1:1,000), followed by an incubation for 1.5 hours with goat anti-mouse HRP conjugates (1:5,000). Target proteins were then detected by chemiluminescence (Roche Diagnostics). Adiponectin, PPARγ and actin protein band densities were assessed and analyzed with ImageJ software. Adiponectin and PPARγ band densities were normalized with that of actin, and the data were represented as fold changes compared to control.

2.6 Statistical analyses

The results are expressed as the means±SEM. Treatments were compared by one-way ANOVA using Tukey's posthoc test to identify statistical differences.

3.   Results

3.1 Cell cytotoxicity determined by MTT assay

MTT assay was used to assess the effects of sorbitol on 3T3-L1 cell cytotoxicity. The results showed that after treatments with sorbitol at the concentrations of 5, 10, 50, and 100 mM, the percentages of viable cells were 90.70±6.23, 84.87±8.47, 91.87±2.83, and 90.0±4.71, respectively, compared to control (Figure 1). Therefore, cell cytotoxicity caused by sorbitol was minimal within the range of concentrations used in the present study.
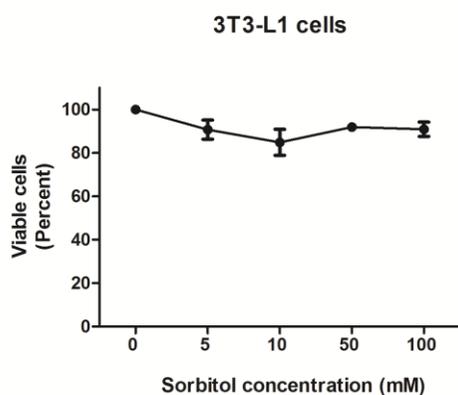

Fig.1. Effects of sorbitol on viability of 3T3-L1 cells, determined by the MTT assay. Pre-adipocyte 3T3-L1 cells were cultured in the presence of sorbitol (0, 5, 10, 50, and 100 mM) for 48 hours. The data are represented as percentages of viable cells compared to control using the following formula: [MTT OD value of sample / MTT OD value of control (cell treated with PBS)] x 100.

3.2   Sorbitol   and   3T3-L1 adipogenesis

3T3-L1 pre-adipocytes were treated with the adipogenesis induction medium (see Methods and Materials). During adipogenesis, fat droplets are accumulated and pre-adipocytes become mature. Therefore, to detect adipogenesis, fat droplets were determined within the cells by using Oil Red O staining. The negative control, the 3T3-L1 pre-adipocytes cultured in media without adipogenesis inducers, showed absorbance of 0.0197±0.0029, while the adipogenesis control, cells with the adipogenesis inducers, showed absorbance of 0.0717±0.0151. This revealed that the adipogenesis control had about 3.6-fold higher absorbance or staining than the negative control, reflecting 3T3-L1 adipogenesis. Additional controls were performed in this study to examine cell responses to adipogenesis activation and inhibition. PPARγ agonist Troglitazone and antagonist GW9662 were chosen because they are known to stimulate and suppress adipogenesis of 3T3-L1 cells, respectively. It was shown that absorbance of Troglitazone-treated cells was 0.101±0.0177, while that of GW9662-treated cells was 0.063±0.014. This reflects about a 40% increases and a 12% decreases in the staining, respectively, compared to the adipogenesis control. For sorbitol-treated cells, the absorbance values were 0.0887±0.0168, 0.077±0.0088, and 0.055±0.0095 for 0.5, 5, and 50 mM sorbitol treatments, respectively. Increasing sorbitol concentration to 50 mM reduced the absorbance. However, there was no significantly difference in absorbance among these sorbitol-treated cells and control ($p>0.05$) (Figure 2).
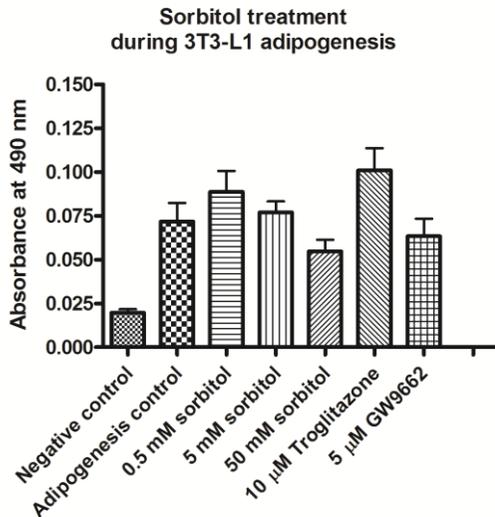
Fig.2. Effects of sorbitol on adipogenesis of 3T3-L1 cells. Cells were plated in 24-well plates and incubated in 10% FBS-containing DMEM without adipogenesis inducers (negative control), with the inducers (control) or with the inducers and treatments. The treatments included sorbitol (0.5, 5, and 50 mM), 5 μM GW9662, and 10 μM troglitazone. Fat droplets within the cells were stained with Oil Red O and the absorbance of extracted dye was measured at 490 nm. Absorbances of Oil Red O in all groups are shown as means±SEM (n=3) after background subtraction.

3.3 Sorbitol and adiponectin synthesis and secretion

First, sorbitol was tested for its effects on adiponectin synthesis. After 3T3-L1 cells were differentiated to mature adipocytes, they were treated with sorbitol (0, 0.1, 0.25, 0.5, 5, and 50 mM) for 24 and 48 hours. Cell lysates and culture media were collected to determine the amount of adiponectin by western blot technique. For cell lysates, adiponectin was detected as monomer at about 30 kDa and β-actin, a loading control, was at about 42 kDa (Figure 3A). The adiponectin/β-actin ratios by sorbitol were calculated as fold change compared to control (Figure 3B). For 24 hour incubation, the fold changes of the

adiponectin/β-actin ratios were 1.050±0.071, 0.936±0.154, 0.953±0.130, 1.073±0.045, and 1.086±0.226 for 0.1, 0.25, 0.5, 5, and 50 mM sorbitol treatments, respectively. The fold changes of the adiponectin/β-actin ratios after 48 hour incubation were 1.1960±0.205, 1.0300±0.211, 1.1600±0.393, 1.1230±0.082, and 0.8833±0.131 for 0.1, 0.25, 0.5, 5, and 50 mM sorbitol treatments, respectively. The results showed that the adiponectin/β-actin ratios in mature adipocytes treated by various sorbitol concentrations were not significantly different from that of control after treatments for 24 and 48 hours ($p > 0.05$).

After that, adiponectin secretion was also analyzed in culture media after 48 hours of sorbitol treatments. Secreted adiponectin were calculated as fold change compared to control and presented in Figure 4. The fold changes of secreted adiponectin were 1.275±0.065, 1.137±0.049, 1.197±0.146, 1.220±0.250, and 1.043±0.269 for 0.1, 0.25, 0.5, 5, and 50 mM sorbitol treatments, respectively. It was shown that sorbitol did not significantly change adiponectin secretion from mature adipocytes, compared to control cells ($p > 0.05$).
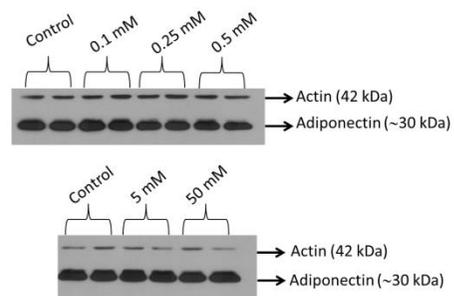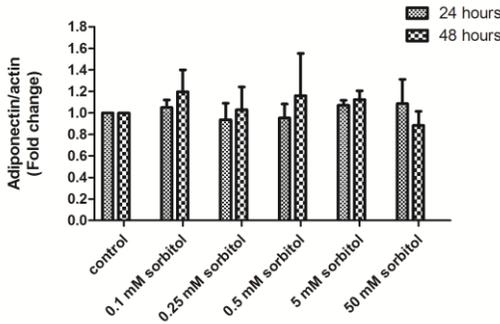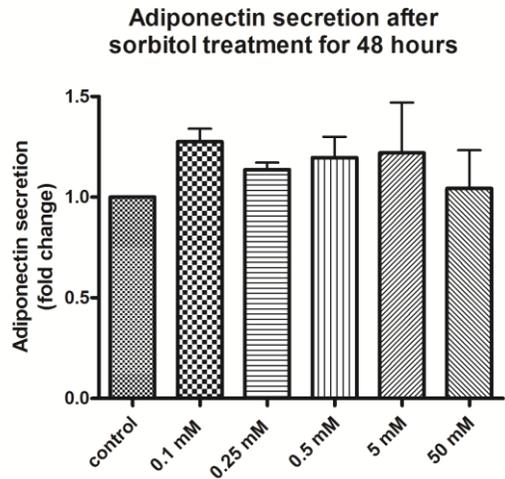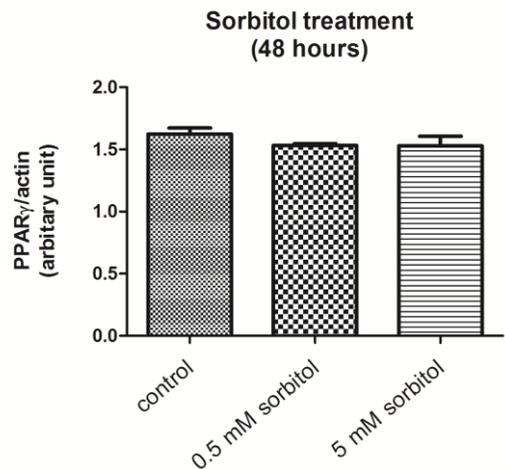

Fig. 3A

Fig. 3B

Fig.3. Effects of sorbitol on adiponectin synthesis in mature adipocytes. Mature 3T3-L1 cells were treated without (control) or with sorbitol (0.1, 0.25, 0.5, 5, and 50 mM) for 24 and 48 hours. Adiponectin mass in cell lysates were determined by Western blot technique. Adiponectin band density (~30 kDa) was normalized with that of β-actin (42 kDa) **(A)** and presented as fold changes of the adiponectin/actin ratios, compared with the control **(B)**. These results are shown as means±SEM from three experiments.

3.4 Sorbitol and PPARγ synthesis

To examine effects of sorbitol on PPARγ synthesis in mature adipocytes, differentiated 3T3-L1 adipocytes were treated with various sorbitol concentrations (0, 0.5, and 5 mM) for 48 hours. Cell lysates were collected to determine the amount of PPARγ by western blot technique. The results are presented as the PPARγ/actin ratios compared to control (Figure 5). It was shown that sorbitol had no significant effects on the PPARγ/actin ratios ($p>0.05$).

Fig.4. Effects of sorbitol on adiponectin secretion. Mature 3T3-L1 cells were treated without (control) or with sorbitol (0.1, 0.25, 0.5, 5 and 50 mM) for 48 hours. Secreted adiponectin in the culture media was determined by SDS-PAGE and Western blot technique. Data were normalized and presented as fold changes, compared to control. These results are shown as means±SEM from three experiments.

Fig.5. Effects of sorbitol on PPARγ gene expression in mature adipocytes. Mature 3T3-L1 cells were treated without (control) or with sorbitol (0.5 and 5 mM) for 48 hours. The amounts of PPARγ protein in mature adipocytes were determined by

Western blot.   PPARγ band density was normalized with β-actin and presented as PPARγ /actin ratio. These results are shown as means±SD.

4.   Discussion

Sorbitol is a food sweetener, widely used as a glucose substitute and metabolized to fructose.   Sorbitol shares similar metabolic pathway to fructose. It has been shown that fructose can induce lipogenesis, insulin resistance, and can increase the risks of diabetes and cardiovascular diseases [10-12]. Therefore, sorbitol was tested whether it would affect adipogenesis and adiponectin synthesis and secretion by using 3T3-L1 cell culture.   Our results showed that sorbitol had no significant effects on adipogenesis and adiponectin syntehsis and secretion.

For adipogenesis assay, fat accumulations in adipocytes were assessed by using Oil Red O staining as a marker of adipogenesis or adipocyte maturation. To verify the system, PPARγ agonist Troglitazone and PPARγ antagonist GW9662 were used because they are known to induce and suppress adipogenesis, respectively. It was found that Troglitazone increased fat accumulation while GW9662 decreased the accumulation. This indicates that the results from our experiments concur with those of previous report [25].

A previous study showed that male Fisher-344 rats fed with diets containing glucose and 10%-20% sorbitol have higher gluocose-6-phosphate   dehydrogenase (G6PD) in epididymal fat tissue [26]. G6PD is an enzyme that maintains levels of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH) in pentose phosphate pathway, facilitating fatty acid synthesis. This result suggested that sorbitol may have direct effects on fatty acid synthesis and hence adipogenesis. Therefore, effects of sorbitol on adipogenesis were examined in the present study. However, our results showed that sorbitol did not significantly affect the adipogenesis of 3T3-L1 cells ($p>0.05$), compared with the control. Even though it was not statistically significant, higher sorbitol concentration tended to reduce the adipogenesis of pre-adipocyte 3T3-L1 cells (Figure 2). This is consistent with previous studies showing that an addition of sorbitol in a diet decreases food efficiency and total body weight gain [26] and also reduces body and abdominal fats [27-28]. Moreover, sorbitol has been shown to be taken up by glia cells via passive diffusion [29] and enter chick embryo heart cells slower than glucose uptake [30].   It is possible that slow sorbitol uptake minimizes the effects of sorbitol on 3T3-L1 cells under used conditions.

Furthermore, previous studies in rat epididymal adipose cells showed that hyperosmotic stress (400-500 mOsm/kg) markedly reduces insulin-induced glucose uptake [31]. Similarly, 3T3-L1 adipocytes pretreated with 600 mM sorbitol strongly decreases the ability of insulin to stimulate glucose uptake, lipogenesis and glycogen synthesis [32].   Sorbitol concentrations chosen in the present study were not high enough to produce hyperosmotic stress, but these concentrations were in the same range as the previous study about its effects on proliferation of human skin fibroblasts and arterial smooth muscle cells [33]. Thereby, we hypothesized that increasing sorbitol concentrations (up to 50 mM) might lower glucose uptake into 3T3-L1 adipocyte and decrease adipogenesis of 3T3-L1 cells. Additional studies are needed to investigate the underlying mechanism.

PPARγ is mainly expressed in adipose tissue, which triggers adipocyte differentiation, and up-regulates adiponectin gene expression [34]. Our study found that the amount of PPARγ protein was not significantly changed by sorbitol. Moreover, mature adipocytes treated with various sorbitol concentrations synthesized and secreted similar amounts of adiponectin to untreated control.   This suggests that

sorbitol does not have direct effects on adiponectin synthesis in adipocytes. This is supported by the study showing effects of ursolic acid on adiponectin. Ursolic acid is an inhibitor of sorbitol dehydrogenase and aldose reductase in polyol pathway; therefore, it inhibits the conversion of sorbitol to fructose. It was shown that ursolic acid reduces blood glucose levels, but does not affect adiponectin levels [35].

5. Conclusion

Sorbitol decreases body and abdominal fat in rat. However, our results showed that 3T3-L1 adipogenesis as well as adiponectin synthesis and secretion from mature adipocytes were not directly affected by sorbitol treatments. Therefore, from this point of view, sorbitol can be used as a sweetener in diets.

6. Acknowledgements

7. References

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