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Stevioside Potentiates Insulin Sensitivity by Elevating Insulin-Stimulated Glucose Uptake in 3T3-L1 Adipocytes

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Abstract: *Stevia rebaudiana* Bertoni is a herb common to tropical and subtropical regions particularly Asia and South America. In countries like Malaysia, the sweet leaves of this plant have been used in daily cooking and as a form of alternative therapy by communities practicing folk medicines. The zero-calorie value of stevia leaves also provide a beneficial sweetening alternative to patients with metabolic syndromes like diabetes and obesity. A cell culture model of 3T3-L1 pre-adipocytes was implemented in this study to investigate the effects of stevioside, a major component extracted from the *S. rebaudiana* Bertoni plant on improving insulin sensitivity. 3T3-L1 pre-adipocytes were first differentiated to mature adipocytes prior to tests. The differentiation process was later confirmed by performing Oil Red-O staining. The degree of insulin sensitivity was measured by performing counts via the radioactive 2-deoxy-[1-$_3$H]-glucose uptake assay. A separate assay was done to estimate the optimum insulin concentration in the glucose uptake of the 3T3-L1 adipocytes. Cells treated with stevioside showed significant increase in glucose uptake at lower concentrations compared to a diabetic drug, rosiglitazone maleate at higher concentrations of the compound. The enhancement of glucose uptake was 2.08-fold (p<0.001) in the cells treated with 30μM stevioside. Stevioside was thus seen to elevate insulin-stimulated glucose uptake concomitant with potentially enhanced insulin signaling in the 3T3-L1 cell culture model.

Keywords: *Stevia rebaudiana* Bertoni, stevioside, glucose uptake, diabetes, insulin sensitivity, insulin resistance, 3T3-L1 cells.

INTRODUCTION

Stevia rebaudiana Bertoni is a perennial herb from the family of *Compositae* which is mainly grown in places with tropical or subtropical climates [1]. It is a day plant with an extensive root system and brittle stems, growing white flowers with light purple throats that normally measures about 1 metre in height [2].The plant has been widely applied in folk and traditional medicine in South American and Asian regions like Brazil, Paraguay, Malaysia and Thailand due to the healing and sweetening properties encompassing its leaves [3]. Interestingly, despite of having such distinct sweetening potencies which exceeds up to 200 times sweeter than normal table sugars, *S. rebaudiana* has little to zero-caloric value as has been reported by Koyama *et al.* (2003) [4]. Thus this plant provides an alternative to normal sugars and is highly beneficial to patients suffering from metabolic disorders like diabetes and obesity, as it sweetens, without elevating their blood sugar levels due to its reported antihyperglycaemic activities [5].

This plant has managed to attract many economic and scientific interests from the worldwide community. Countries like Japan has been marketing stevioside as an alternative sweetener in the food and drug industry for years, and is in fact, one of the first countries to do so. The application of stevioside in the food and drug industries has been vast, as they were introduced in foods like gums, drinks, sauces, and candies, and also in mouthwashes and toothpastes [6].

The sweetening properties of *S. rebaudiana* were mentioned to be induced by compounds extracted from the plant, called steviol glycosides. Some of the steviol glycosides that have been successfully extracted were rebaudioside A, rebaudioside C, dulcoside, and also stevioside, being the major constituent [2]. A single stevioside compound is built up by three molecules of glucose, and an aglycone called steviol, making it a diterpenoid glycoside as a whole [6]. Steviol glycosides with the likes of stevioside have been found to be non-toxic and non-mutagenic, with no distinct side-effects, post-consumption [2].

It was asserted that *S. rebaudiana* gives out many therapeutic benefits, but studies on the cellular and molecular levels are still lacking. Effects of food components towards cells, and more importantly the molecular interactions exchanged between the two, are essential in understanding and confirming the many health benefits, a food product can be asserted to have. Thus the more commonly consumed stevioside, a major component from the ***S. rebaudiana*** is of no exception.

In this study, the effects of stevioside on insulin sensitivity will be investigated through the assays of glucose uptake on the 3T3-L1 adipocytes, as an *in vitro* cell culture model. 3T3-L1 cells is one of the most highly applied cell

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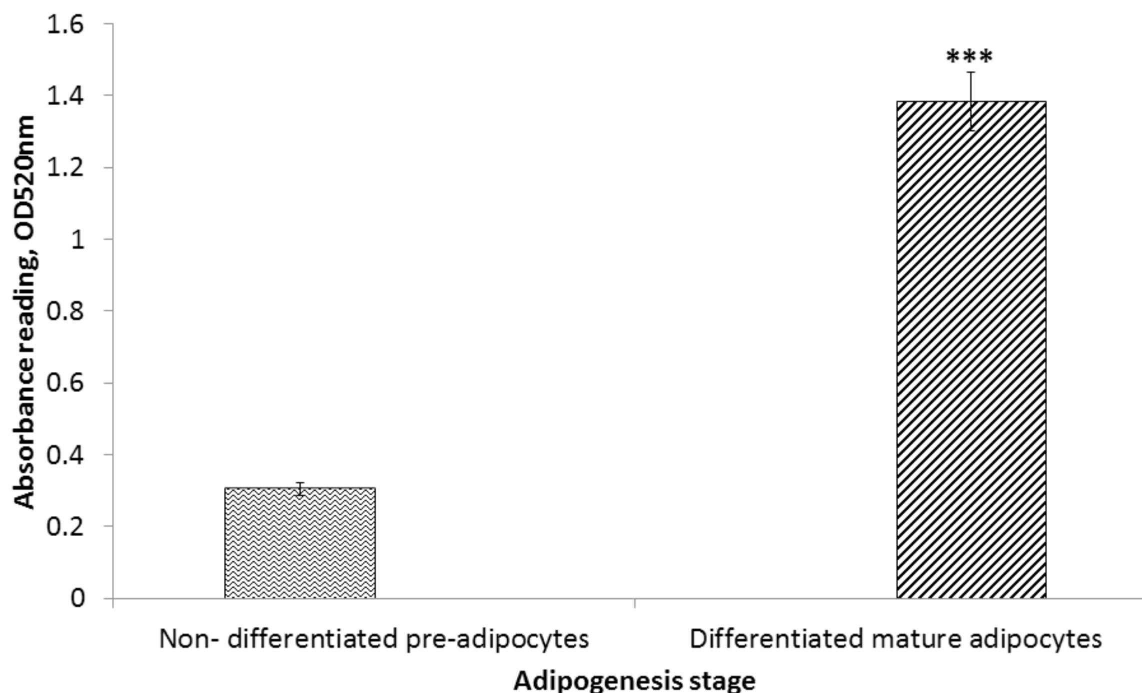

Fig. (1). Data shows the elevation in absorbance readings in 3T3-L1 adipocytes at different levels of adipogenesis via Oil Red-O staining. Statistically significant difference was presented as ***p<0.001.

lines in the studies concerning insulin sensitivity and insulin resistance as they are more cost effective and easier to handle compared to freshly isolated cells [7]. The glucose uptake level of the adipocytes will be observed post-treatment with stevioside and by implementing the radioactivity counting of 2-deoxy-[1-^{3}H]-glucose that had been taken up during the course of the experiment. It was hypothesized that the glucose uptake levels will be elevated in the 3T3-L1 cells treated with stevioside, together with the stimulation from insulin.

METHODS

Cell Culture and Differentiation

3T3-L1 pre-adipocytes were purchased from American Type Culture Collections and were cultured in Dulbecco's Modified Eagle's Media (DMEM) supplemented with newborn calf serum. Cells were grown and incubated in the culture chambers at a specific temperature and carbon dioxide saturation. Upon inducing the differentiation process, the cells were seeded into plates and were supplemented with insulin, dexamethasone, (DMX) and 3-isobutyl-1-methyl-xanthine in DMEM before further incubation commenced [8].

Oil Red-O Staining

Powdered Oil Red-O was purchased from Sigma Aldrich and was later dissolved and filtered appropriate for cell culture applications. 3T3-L1 cells in 6-well plates were fixed with 10% formalin and were later washed with phosphate buffered saline (PBS). The cells were then stained with the filtered working solution of Oil Red-O for 10 minutes. Following the staining process, cells were washed with distilled water to remove excess staining and then viewed under the microscope. Lipid stainings on the cells were later eluted with isopropanol and their absorbance were read on the spectrophotometer at a wavelength of 520nm.

Glucose Uptake Assay

Matured 3T3-L1 adipocytes on 12-well plates were serum starved for two hours before being washed with the Kreb's Ringer bicarbonate (KRB) buffer. Next, cells were treated with stevioside and rosiglitazone maleate (AVANDIA), an antidabetic drug acting as a positive control at concentrations of 30 to 150 μM, This was coupled with the acute stimulation from insulin and then pre-incubated before the addition of 2-deoxy-[1-^{3}H]-glucose to initiate glucose uptake. After 1 hour, cells were washed with ice cold KRB buffer and dissolved with 0.1% sodium dodecyl sulphate (SDS). Dissolved samples were then collected and added with scintillation cocktail and analysed on the liquid scintillation counter (LSC).

RESULTS

As initially expected, the 3T3-L1 pre-adipocytes were successfully differentiated into mature adipocytes. This was confirmed by performing the Oil Red-O staining procedures onto the cells, both before and after the induction to differentiation. Fig. (**1**) shows the differences in absorbance readings in the stains of undifferentiated and fully-

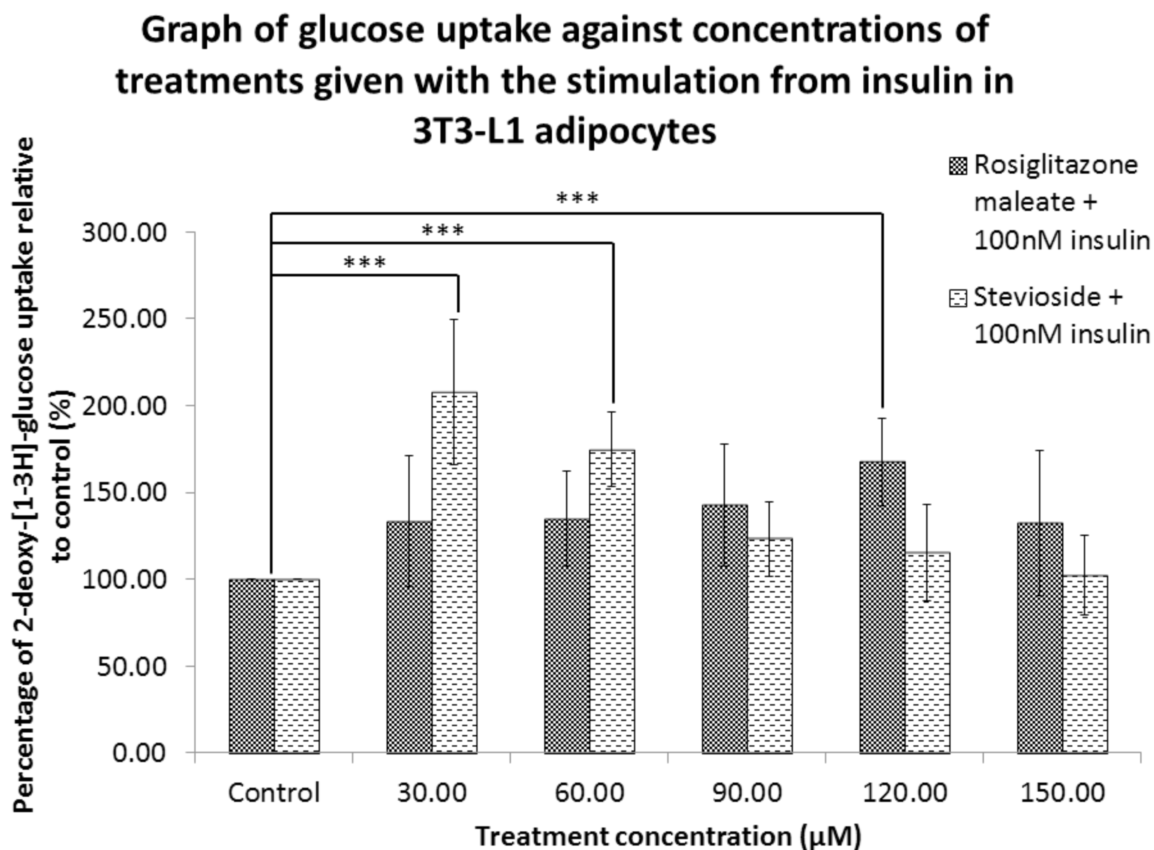

Fig. (2). The bar chart represents the percentage of 2-deoxy-[1-^{3}H]-glucose uptake against rosiglitazone maleate and stevioside concentrations in 3T3-L1 adipocytes, with the presence of insulin. Values presented are mean ± standard error mean (SEM) from three replicates. Statistically significant data are given as ***$p<0.001$ compared to control.

differentiated adipocytes. The eluted stains were measured on the spectrophotometer for their absorbance values and it was found out that those cells that had been differentiated into mature adipocytes gave an average reading of 1.384 which was 4.54-fold higher than the undifferentiated pre-adipocytes.

From the glucose uptake assay that was conducted, several elevations in glucose uptake were observed *in vitro* in the 3T3-L1 adipocytes. Based on Fig. (**2**), it was shown that there was a maximum increase in the 3T3-L1 adipocytes treated with 30 μM of stevioside, which was observed to be at 2.08-fold higher than control. This also exceeds the maximum uptake in the cells treated with rosiglitazone maleate, which was at 120 μM giving an elevation of only 1.68-fold compared to the control group.

DISCUSSIONS

Oil Red-O staining is one of the common tests conducted in assessing levels of adipogenesis by staining the lipid droplets, formed in mature adipocytes [9]. Such staining methods are one of the most widely applied in many investigations involving 3T3-L1 adipocytes. Fig. (**1**) shows significant increase in the absorbance reading of fully-differentiated 3T3-L1 adipocytes, showing high levels of lipid staining, which is clearly indicative to mature adipocytes and not to pre-adipocytes. The high lipid accumulations were also shown in the thick viscosities in the media of the differentiated adipocytes as observed by Zebisch *et al*. (2012) [10]. This concludes that the IBMX-insulin-DMX cocktail used in the procedures managed to initiate the differentiation process as they either elevate the expressions and activities of CAAT/enhancer binding proteins (C/EBPs) or peroxisome proliferator receptor γ (PPARγ) or both, which are responsible in the process of adipocyte differentiation [11].

Once the differentiation process was confirmed, the cells can therefore be implemented in the glucose uptake assay. Based on Fig. (**2**), it was clearly evident that stevioside has the ability of increasing the 2-deoxy-[1-^{3}H]-glucose uptake *in vitro* as seen in the fat cells. The fact that stevioside increases extracellular glucose uptake, is also coherent with the findings from Gregersen *et al*. (2004) [5], of which stevioside lowers glucose levels in diabetic patients. The significance behind such indicative results is that when glucose uptake levels were observed to have increased, this also shows that the amount of extracellular glucose taken up by the cells were higher thus will most probably lower blood glucose levels, if observed in the normal clinical settings. Rosiglitazone maleate, a drug commonly prescribed for diabetics which promotes antihyperglycaemic activities, was used as a control in this study and the results demonstrates that the actions of stevioside even managed to surpass that of this drug, representative of its effectiveness as an antihyperglycaemic agent.

CONCLUSION

In accordance with these findings, it was safely stipulated that stevioside can potentiate insulin-stimulated glucose

uptake, *in vitro* as showcased by the 3T3-L1 cell culture model. Thus, stevioside might hold promises in improving insulin sensitivity, and unravelling diabetes as a whole. Although the exact pathways of which stevioside may have acted on in the cellular level remains unknown, stevioside can still be suggested as an adjunctive antihyperglycaemic agent in the treatment of diabetes. Nevertheless, more in-depth studies need to be conducted to confirm and uncover these potentials of stevioside and *S. rebaudiana* even further.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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