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Early Investigation on the Effects of Stevioside on Differentiation Process of Fibroblast into Adipocytes

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Abstract. Obesity is one of major health problems occurred worldwide including Malaysia. Unfortunately, no suitable treatment or mechanism can be used to solve the problem effectively. In order to find the most appropriate treatment, stevioside was chosen to investigate its effects in preventing obesity. Stevioside was used to observe its effect in four stages of differentiation process (lipid formation) 3T3-L1 murine pre-adipocytes (fibroblasts) into adipocytes. The process was observed using Oil red-O staining and measured using a spectrophotometer at the wavelength of 520 nm. From the results, stevioside was significantly inhibited the differentiation process at stage 2. Stevioside may interfere with the other supplements added in the cell culture media, which triggered the inhibition. However, the mechanism was unclear and required further studies.

1. Introduction

Obesity remains the major health problem in Malaysia (Lim, 2016). Until now, none effective treatments are available to solve the problem. The best practices are by taking an appropriate and healthy diet and do a regular and consistent exercise. Beside the development in preventing, managing and controlling obesity, various researches are being conducted to find the most possible methods and treatment to resolve the challenge. One of the efforts is by elucidating a potential of stevioside, a natural sweetener, in preventing the excess gain.

Stevioside is a bioactive compound extracted from the leaf of *Stevia rebaudiana*. The herb is widely available in the northeastern region of Paraguay, Brazil, Argentina, Europe and Asia including in Malaysia (Tavarini & Angelini, 2013). Stevioside has been used as a natural sweetener to replace table sugar, which is known to cause various health complications. Meanwhile, the natural sweetener is zero calorie and has been used traditionally to treat diabetes in Paraguay (Mohd-Radzman et al., 2013a). However, its effect in preventing obesity is still unknown and will be evaluated in this study.

Physiologically, obesity occurs when size and volume of adipocytes in adipose tissue are increasing unlimited because of high glucose uptake and lipogenesis (González-Muniesa et al., 2017). In order to control the obesity from the beginning, an effective treatment may be evaluated its effects on adipocyte formation. Adipocyte formation involves differentiation procedure from fibroblast cells to adipocytes. In order to access potential stevioside in controlling obesity, differentiation process of 3T3-L1 murine adipocytes was measured with and without the stevioside

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treatment.

2. Materials and Methods

2.1. Sample preparation

Stevioside was purchased from Sigma-Aldrich Co. (Germany) and prepared in the concentration of 30µM (Mohd-Radzman et al., 2013) prior to adding in the four different stages of adipocyte differentiation i.e. stage 1, 2, 3, 4 (Ismail et al., 2013 and Mohd-Radzman et al., 2013b).

2.2. Cell culture and differentiation

3T3-L1 pre-adipocytes or also known as fibroblasts were cultured in Dulbecco's Modified Eagle's Media (DMEM) according to Ismail *et al.*, 2013 and Mohd-Radzman et al., 2013. For the differentiation procedures, the cells were cultured in the DMEM supplemented with insulin, dexamethasone (DMX) and 3-isobutyl-1-methyl-xanthine (IBMX).

2.3. Oil red-O staining

The cells were washed with phosphate-buffered saline (PBS) before being fixed with a solution of 10% formaldehyde in PBS for overnight. The solution was discarded prior to introduce the cells with the Oil Red-O dye for 10 minutes in room temperature. Then, the dye was removed with 100% isopropanol before the cells were measured using a spectrophotometer at 520 nm (Mohd-Radzman et al., 2013).

2.4 Statistical analysis

Data were presented as mean ± standard error mean (SEM) before proceeded with one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. The analysis was conducted using the Sigma Plot version 16 software. Statistically different means were acknowledged at $p < 0.05$.

3. Results

3T3-L1 pre-adipocytes (control) were successfully and significantly differentiated into adipocytes (untreated) (Figure 1). After the cells treated with stevioside at every stage of adipocyte differentiation, the lipid content in the adipocytes was changed compared to the untreated cells. Stevioside was found to inhibit adipogenesis process significantly at stage 2 compared to the other stages. Meanwhile, the compound found to stimulate the fat formation in stage 1, 3 and 4.

4. Discussion

3T3-L1 adipocytes were used in the study because the cells are well-known as a cell culture model to study obesity and diabetes (Knutson and Balba, 1997). In addition, the cells have the ability to imitate fat cell formation in animal and human. Basically, adipocytes are the major component in adipose tissue; our lipid storage. Investigation and observation on the cells before and after some treatments may give a clue in controlling excess weight gain.

Fat cell formation or also known as differentiation procedure can be conducted *in-vitro* through four different stages (Ismail et al., 2013). Each stage requires different supplements to support the growth. In stage 1, the cells were cultured in medium 1, which contained DMEM with newborn calf serum (NCS) (10%), L-glutamine (2 mM), antibiotic/antimycotic (1% of 100x) and gentamicin (0.1%) to maintain 3T3-L1 fibroblast growth. DMEM was used as a base medium. Addition of NCS in the medium because it has a low level of insulin-like growth factor (IGF), which allows cells to grow as fibroblasts and prevents them from differentiating (Knutson and Balba, 1997). However, with additional of stevioside, the cells seem able to differentiate into adipocytes and hinder the effect of NCS.

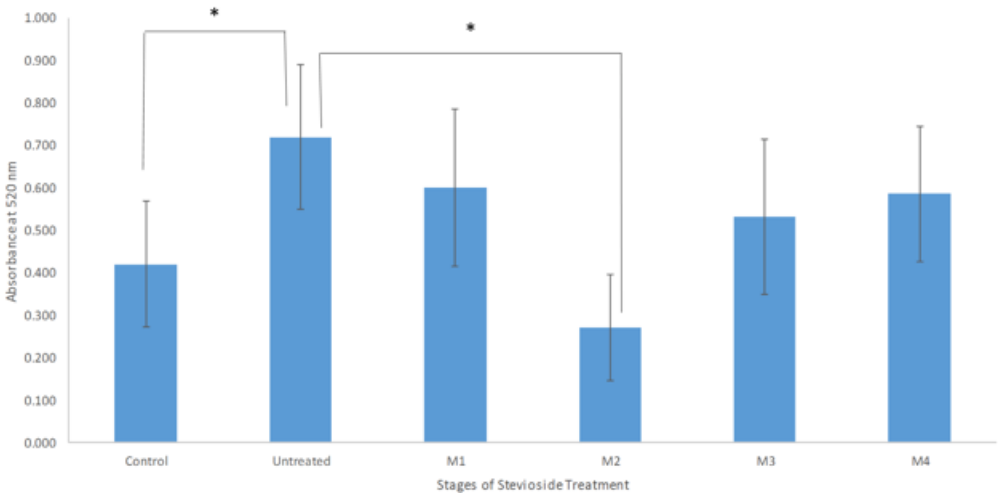


Figure 1. Optical density of lipid formation with and without treatment with stevioside (30µM) after stained with Oil Red-O dye and measured using spectrophotometer at 520 nm. Control: 3T3-L1 pre- adipocytes (fibroblast), Untreated: 3T3-L1 adipocytes after differentiation process without stevioside's treatment, M1: 3T3-L1 pre-adipocytes in stage 1 of lipid formation, M2: 3T3-L1 pre-adipocytes in stage 2 of lipid formation, M3: 3T3-L1 pre-adipocytes in stage 3 of lipid formation and M4: 3T3-L1 pre-adipocytes in stage 4 of lipid formation. Mean ± SEM (n = 6). Statistically significant differences with $p < 0.05$ (ANOVA and Tukey's posthoc test).

In stage 2, the cells were cultured in medium 2, which is similar to medium 1 except NCS was replaced with FCS (fetal calf serum) (10 %) and addition of DMX (0.25 µM), IBMX (0.5 mM) and insulin (166 nM). FCS has a high level of IGF, which stimulates the differentiation of pre-adipocytes into adipocytes. Meanwhile, DMX and IBMX were used to induce differentiation of 3T3-L1 pre- adipocytes through the activation of transcription factors, the CCAAT/enhancer-binding proteins (C/EBPs) and the PPARγ. Insulin was also added to the culture medium to accelerate the differentiation procedure in the cells (Knutson and Balba, 1997). The most effective differentiation process should occur at the stage. Interestingly, stevioside is able to inhibit the lipid formation hereby decreasing the differentiation process (Figure 1). The possibility of the mechanism is the stevioside may interfere with effects of FCS added in the medium. FCS contains a high level of IGF, which plays important role in adipocyte formation and has proteins sequence similar to insulin (Ismail et al., 2013). Meanwhile, stevioside has similar function to insulin (umpublished data), which can mimic the insulin hormone. The existence of both stevioside and IGF may disrupt the role of IGF, thus inhibited the fat formation. However, the notion is required further investigation.

In stage 3 and 4, the cells were grown respectively in medium 3 and 4. Chemical compositions in the medium 3 was similar to components in medium 2 except without DMX and IBMX addition. Meanwhile, medium 4 was prepared as according to medium 3, but without insulin to maintain the adipocyte differentiation (Knutson and Balba, 1997). Stevioside is found to stimulate the differentiation process at both stages. According to the results, the potential of stevioside in controlling obesity is still unclear. The finding shows the ability of the compound in inhibiting lipid accumulation at stage 2 compared to the other stages. However, the reaction between chemical and supplements used in the media may interfere with the results. Thus, more studies need to be conducted such as by using an obesity animal model with and without the stevioside consumption to observe its effects as an anti-obesity agent.

5. Conclusion

Potential stevioside in controlling obesity is still uncertain even it shows positive results at stage 2 of the differentiation procedures. Other studies should be done to confirm the investigation.

6. References

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