

## Effect of allithiamine on the level of hyperglycaemia-induced advanced glycation end products

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### SUMMARY

*Diabetes mellitus is a rapidly growing public health burden in both developed and developing countries. Diabetes mellitus is accompanied by hyperglycaemia, which can cause tissue injury by several mechanisms. One of these is the formation of advanced glycation end-products (AGEs). In this study, the effect of allithiamine, a fat-soluble thiamine derivative, was investigated on hyperglycaemia-induced AGEs levels using human umbilical cord vein endothelial cells (HUVECs) as a hyperglycaemic model. HUVECs were isolated by enzymatic digestion, characterized by flow cytometer and treated 30 mM glucose plus allithiamine or thiamine or cell maintenance medium as control. Allithiamine was synthesized and purified. The structure of the synthesized and isolated compound was verified by reverse phase HPLC and MALDI-TOF. AGEs were evaluated by ELISA. Collectively, our results indicate that allithiamine can reduce level of the hyperglycaemia-induced AGEs similar to thiamine.*

**Keywords:** allithiamine, hyperglycaemia, advanced glycation end-products, endothelial cells

### INTRODUCTION

Thiamine, known as vitamin B1, has pivotal role in energy metabolism (Lonsdale, 2006). Thiamine is the main compound of thiamine pyrophosphate which is a cofactor of enzymes involved in glucose metabolism. Allithiamine is a lipid soluble form of vitamin B1, which was found firstly in *Allium* genus (Fujiwara et al., 1954). In our preliminary experiment, the effect of allithiamine was investigated in streptozotocin-induced diabetic mice. The results confirm the improvement of neuropathic pain sensation (Biro et al., 2018).

Diabetes mellitus (DM) is a metabolic endocrine disorder with serious micro- and macrovascular complications. The latest prognosis of the World Health Organization (WHO) predicted an increasing prevalence of DM. DM is accompanied by hyperglycaemia, which may cause tissue damage by several mechanisms (Berrone et al., 2006). One of the major metabolic route is the nonenzymatic glycation of intra- and extracellular proteins, lipids, nucleic acids resulting in advanced glycation end-products (AGEs) (Nin et al., 2011). Increased level of AGEs leads to harmful intermediates causing enhanced oxidative stress and consequential inflammation (Zhang et al., 2003). Several studies suggest that the increased formation of AGEs is major cause of endothelial dysfunction in DM. It has been suggested that unphosphorylated thiamine and its synthetic derivatives can alleviate endothelial dysfunction by inhibiting AGEs pathway (Balakumar et al., 2010; Brownlee, 2005). The object of this study was to examine effect of allithiamine compared to thiamine in primary cultured human umbilical cord vein endothelial cells (HUVECs) as hyperglycaemic model investigating level of AGEs.

### MATERIALS AND METHODS

#### Preparation, purification and verification of pure allithiamine

Synthesis of allithiamine was carried out based on the experiments by Biro et al. Briefly, allyl thiosulphate resulting from the reaction of allyl chloride with sodium thiosulfate was reacted with thiamine hydrochloride with open thiazole ring resulted in different thiamine derivatives. Thiamine derivatives were separated using reversed phase HPLC system. Measurements were carried out using Merck-Hitachi LaChrom liquid chromatograph, equipped with diode array detector L-7455, automatic sampler L-7250 interface L-7000, pump L7100 and HPLC System Manager Software. Allithiamine was analysed on a LiChroCART® 250-10 RP-18 column (12 µm) using gradient elution. MALDI spectra of allithiamine samples were obtained in positive-ion mode using a Bruker Biflex MALDI-TOF mass spectrometer equipped with delayed-ion extraction.

#### Cell culture and allithiamine treatments

The human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords. Umbilical cords were obtained from Department of Obstetrics and Gynaecology. Enzymatic digestion of HUVECs was performed by 22U/100mL collagenase solution incubating for 15 min at 37 °C. HUVECs were grown using M199 medium supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 1% Amphotericin B, 2 mM glutamine and Endothelial Cell Growth Medium-2 (EGM-2) at 37 °C in a humidified incubator under 5% CO<sub>2</sub>. Media was changed every two days until cells reached around 90% confluency. At confluency, cells were either subcultured or used for experiments. Media as described above was used as control and had 5.6 mM D-glucose. For high glucose treatment, D-glucose was added to M199 for a final

concentration of 30 mM. Cells were divided in four groups: 24 h incubating with 5 mM glucose (Control), 24 h incubating with 30 mM glucose (HG), 24 h incubating with 30 mM glucose then 15  $\mu$ M allithiamine was added (HG-AT) and 24 h incubating with 30 mM glucose then 50  $\mu$ M thiamine (HG-BT) was added. Cells were incubated for one week prior to determination of AGEs formation.

### Flow cytometry studies

For identifications of HUVECs, flow cytometry measurements were performed. Cells were incubated with four kind of antibody labelled by fluorescent dye for 30 minutes at room temperature in dark. Approximately 100,000 cells were used for one measurement. Cell counting was performed using a Bürker chamber. FITC-labelled mouse anti-human CD31, PE-labelled mouse anti-human CD54, APC-labelled mouse anti-human CD106 and PerCP-Cy5.5 labelled mouse anti-human CD45 was used for labelling. The measurements were carried out using a Becton Dickinson FACS Aria<sup>TM</sup> III Cell Sorter (Becton Dickinson, Mountain View, CA, USA). Circa 20000 cells/sample were recorded and analysed. Cytofluorimetric data were analysed by using FCS Express 4 Research Edition (De Novo Software, Glendale, CA, USA).

### Measurement of advanced glycation end-products

The assay was performed according to the manufacturer's instructions using OxisSelect<sup>TM</sup> Advanced Glycation End Product (AGE) Competitive ELISA Kit (Cell Biolabs Inc., San Diego, California). Protein determination required for normalization were determined in the cell lysate using Pierce<sup>TM</sup> BCA Protein assay (Pierce Biotechnology, Rockford, USA). All measurements were performed with the microplate reader (SPECTROstar<sup>Nano</sup>, BMG Labtech, Ortenberg, Germany).

### Statistical analysis

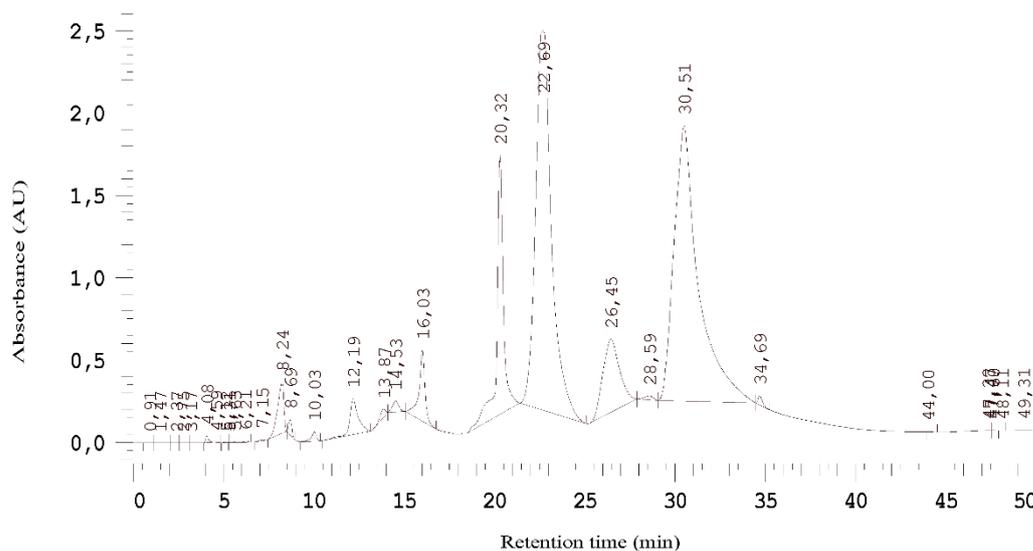
For multiple comparisons, results were analysed by ANOVA followed by modified t-test for repeated measures according to Tukey's method. Data were presented as mean $\pm$ SEM. P<0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

### Verification of pure allithiamine

Preparation of allithiamine resulted in a wide variety of thiamine and other by-products as we experienced. Derivatives were separate by HPLC-method. Allithiamine was eluted at 30.51 min (shown in *Figure 1*), as we identified with MALDI-TOF (data not shown).

Figure 1: HPLC chromatogram of allithiamine

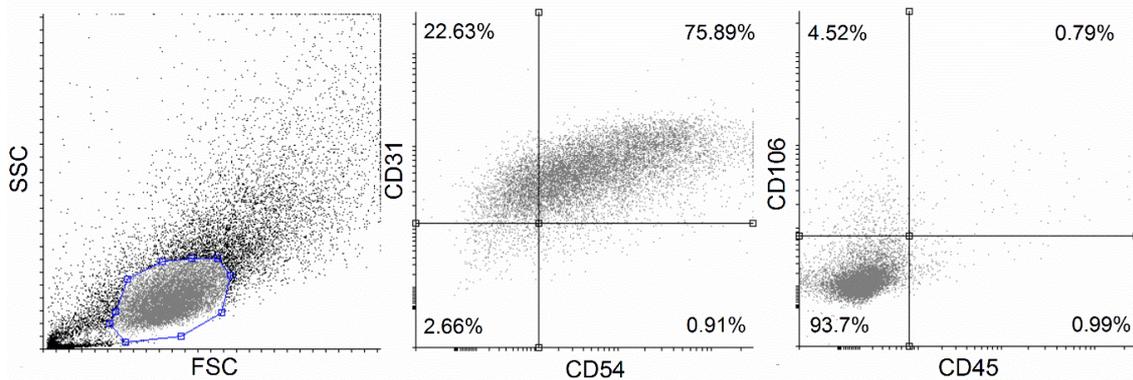


### Characterization of HUVECs by flow cytometer

Isolated HUVEC cells were characterized for positive and negative marker expression using flow cytometry and antibodies against specific marker proteins: CD54, CD31, CD45 and CD106. As it is shown in *Figure 2* isolated HUVECs showed high

CD54 and CD31 positive marker expression levels (app. 76% double positive cells) while app. 94% of cells did not express CD45 and CD106 markers strongly suggesting the high accuracy and efficiency of cell isolation.

Figure 2: Flow cytometry analysis of HUVEC cells

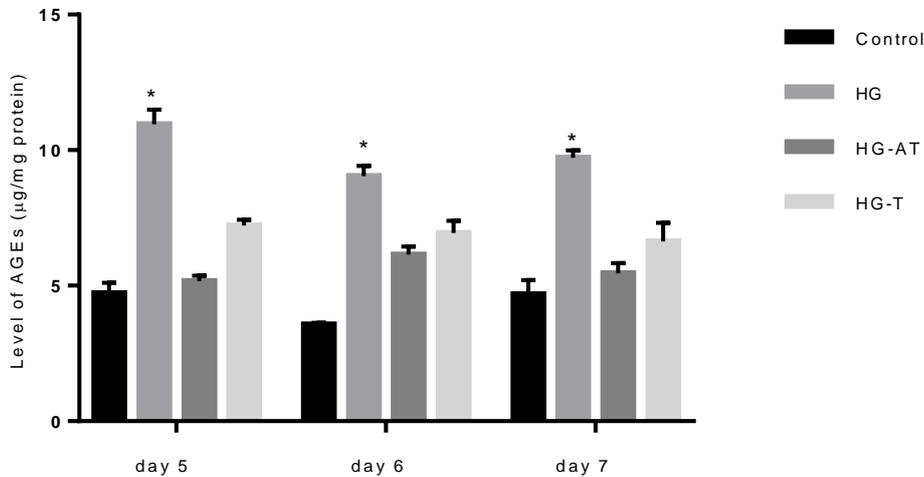


**Level of advanced glycation end-products (AGEs)**

AGEs are one of the most important pathogenetic factor in the development and progression of both microvascular and macrovascular damages (Brownlee, 2001). AGEs were measured in the cells incubated with 5 mM glucose, 30 mM glucose, 30 mM glucose and 15 μM allithiamine or 50 μM thiamine. Amount of AGEs were evaluated after one-week incubations in all

groups. The data of the 5- 6- and 7-day incubations were shown on the Figure 3. Positive correlation was shown in the levels of AGEs and hyperglycaemia. Both allithiamine and thiamine alleviate the hyperglycaemia-induced increase of AGEs significantly. \* indicates significant difference on level of AGEs between HG and Control, HG-AT, HG-BT group on the figure.

Figure 3: Level of AGEs after one week of incubation with the allithiamine and thiamine



**CONCLUSION**

The object of this study was to examine the effect of allithiamine compared to thiamine in primary cultured human umbilical cord vein endothelial cells as hyperglycaemic model investigating level of advanced glycation end-products. We found that allithiamine has a beneficial effect on the level of hyperglycaemia-induced AGEs similar to thiamine. As it is known, thiamine is a cofactor of enzymes involved in glucose metabolism (*pyruvate dehydrogenase, transketolase, α-ketoglutarate-dehydrogenase*) which may play a role in its positive effects (Kern et al., 1997). Chemical

structural similarity suggest that allithiamine may have an analogue influence on glucose metabolism. It is assumed that allithiamine is able to increase the activity of these enzymes with particular regard to *transketolase* activity. Increasing *transketolase* activity has been shown to reduce the flux of metabolic pathways involved in the pathomechanism of hyperglycaemia (Nishimura, 1998); (Gerald and King, 2010) including the AGE-pathway. As regards particular mechanisms of action and possible clinical value, further investigations are needed to confirm our findings.

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