EVALUATION OF PANCREATIC AND EXTRA PANCREATIC EFFECTS OF BRANCHED AMINO ACIDS

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Abstract

Background and aims: Leucine, Isoleucine, and Valine collectively known as Branched-chain amino acids (BCAAs), can be closely associated with metabolic dysregulates and with insulin resistance. We aimed to explore the role of BCAAs as potential treatment option for diabetes. Material and method: Bioassay the effect of BCAAs on MIN6 cell line on insulin secretion and pancreatic beta cells expansion, then were checked for inhibitory potential of pancreatic amylase, glucosidase and lipase as alternative approach for diabetes treatment. Results: BCAAs significantly enhance insulin secretion parallel to L-alanine efficacy. Furthermore, BCAAs obtain a dose dependent β-cell proliferation similar to glucagon-like peptide-1. Moreover, these acids could restore the secretory function of MIN6 β-cell despite stressful gluco-lipo-toxicity; separately or combined. Moreover, BCAAs exerted a dose dependent dual inhibition of amylase, glucosidase and lipase. Conclusions: Our current findings suggest that BCAAs supplementation may have a potential therapeutic effect against diabetes as insulin releasing agent and as specific inhibitors for both α-amylase/α-amylloglucoside and lipase

key words: BCAAs, insulin secretion, beta cells proliferation, amylase, glucosidase and lipase

Background and aims

In type 2 diabetes mellitus (T2DM) the biphasic pattern of insulin secretion, consider a very sensitive marker for normal beta cell function. The early phase of releasing the insulin last for 10 minutes, followed by the second phase, which is slower, more progressive and lasts as long as the glucose infusion [1]. It has been documented in T2DM after glucose infusion, the early phase abolished and the late phase delay [1]. The impairment of the first phase of insulin secretion is a significant marker for islet dysfunction in T2DM. Furthermore, data show that any restoring of the insulin level during the first phase, is consider pivotal in the transition from impaired glucose tolerance to normal glucose tolerance in diabetic subjects [2]. The insulin secretion can be induced by incretin-dependent mechanisms, which include
two major incretin hormones, glucagon-like peptide-1 (GLP-1) and the glucose-dependent insulinotropic peptide (GIP), furthermore glucose and certain amino acids can enhance the insulin releasing mechanism indirectly via stimulate the releasing mechanism of GLP-1 secretion through elevated the concentration of cytosolic Ca\(^{2+}\) in the intestinal cells [3]. Moreover, increasing the stimulation of insulin secreted by glucose and amino acids also could achieved directly [4]. for example, glutamine can directly enhance insulin secretion from β-cells, by activating glutamate dehydrogenase, allowing for the glutamate to convert to α-ketoglutarate, after glutamine become glutamate by glutaminase in the cytosol, which allows the α-ketoglutarate to enter the TCA cycle to give ATP, activating the insulin secretion pathway [5]. Some of the amino acids may enhance the insulin secretion, by serving as potent glucagon secretagogues, causing an increase in the glucose level in the blood, which then releases the insulin by β-cells [6].

In other hand, The BCAAs have different physiological roles regarding protein synthesis, enhancing insulin resistance, proliferation and hindering apoptosis in lymphocyte and hepatocyte, respectively. Furthermore, it can affect the secretion of neurotransmitter in the brain [7]. Moreover, BCAAs could reduce the anaerobic respiration improving the muscles endurance for fatigue in heavy exercise [8].

We aim in this study to evaluate the effect of BCAAs on insulin secretion and proliferation of pancreatic beta cell, as well as the effect of these acid on extra pancreatic digestive enzymes such as amylase and lipase as alternative therapeutic approaches for T2DM.

Material and method

MIN6 cell line culture

The fresh passage of MIN6 β-cells were cultured in Dulbecco Modified Eagle Medium (DMEM) with 15% fetal bovine serum (FBS) and penicillin (100 μg/mL), in a 37°C humidified atmosphere with 5% CO\(_2\). The culture medium was changed every 48 h [9]. All the material in tissue culture was obtained from Invitrogen (USA). All other chemicals and solvents used in this article were purchased from Sigma-Aldrich (St. Luis, MO, USA). Unless stated otherwise.

Insulin secretion static incubation experiments

MIN6 cells were seeded in 96-well plates at density 50,000 cell/well. At the day of the experiment, DMEM culture medium was removed followed by washing the cells with phosphate-buffered saline (PBS). Cells were incubated again for 1h at 37ºC in 5% CO\(_2\) in Krebs-Ringer phosphate buffer (KRH) composed of (mM)129 NaCl, 5 NaHCO\(_3\), 4.8 KCl, 1.2 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 10 HEPES, 2.5 CaCl\(_2\) and 0.1% BSA (pH 7.4, NaOH) supplemented with 1.1 mM glucose. After 1h the KRH was removed, and the cells were washed in glucose-free KRH. Subsequent test incubations in 5.6 mM glucose - KRH alone as untreated negative sample or supplemented with (L-alanine 10 mM) as classical and robust insulin secretagogue [10] and with Ile, Leu, Val and BCAAs combination, which were procured from Santa Cruz (USA), at concentrations (1-25mM) for 1h at 37 ºC. Then the medium was collected and stored at -20ºC for a subsequent ELISA determination Kit of the amount of secreted mouse insulin, which was purchased from ALPCO (USA). Cell viability was assessed after 1h using Sulforhodamine B (SRB) colorimetric assay [11]. Which was purchased from Promega
When investigating the effects of extracellular Ca\(^{2+}\)– free incubations on pancreatic insulin secretion \[10\], 2.5 mM CaCl\(_2\) was removed from KRH buffer preparations, so that cells were incubated in a Ca\(^{2+}\)– free KRH in the same panel of glucose stimulated insulin secretion studies described previously.

**Pancreatic beta cells under chronic exposure to high concentrations of glucose and free fatty acids**

Glucotoxic media contain 22.2 mM of glucose concentration. Lipotoxic media contain 0.25 mM of palmitic acid Glucolipotoxic media contain of both D-glucose and palmitic acid at previous concentration \[12\]. MIN6 cells were cultured for 24 h at 37 oC in respective media. Then each of these media were replaced with KRH buffer then incubated with 10 mM alanine or selected concentrations of Ile, Leu, Val, and BCAAs combination as for 1h at the same condition. Finally, the concentration of secreted insulin was measured using ELISA determination kit and Cell viability was assessed using SRB assay.

**Cell proliferation assays of MIN6 cells**

MIN6 cells (fresh passage) were cultured with DMEM without FBS onto 96-well plate (10,000 cell/well density) for 24 h. then were treated with different concentrations of Ile, Leu, Val, or BCAAs or (GLP-1 at 500 nM; the classical robust proliferative incretin) along with Bromodeoxyuridine (BrdU) dye (10 μM/well) for 24h. The rest of the assay was carried out to the manufacturer’s protocol instructions of BrdU ELISA kit \[10\]. Which was procured from Roche Diagnostics (Germany). For viability assay, MIN6 cells were seeded on 96-well plates (10,000 cells/well) in growth medium with different concentrations of Ile, Leu, Val, BCAAs combination. After 48-h incubation, the effects of these acids on cell viability were evaluated by SRB assay.

**Enzymatic starch digestion assay**

The extent of polysaccharide breakdown into glucose was measured to evaluate the dual inhibition of alpha amylase (EC 3.2.1.1) / alpha glucosidase (EC 3.2.1.20) under the effect of BCAAs at (0.5 – 10 mM). Acarbose at (100 μg/mL) was also used as reference drug against both alpha amylase and alpha glucosidase, which was procured from Santa Cruz (USA), the concentration required for starch digestion 50% inhibition was calculated (IC50 value).

**Spectrophotometric quantification of pancreatic lipase (PL) (EC 3.1.1.3)**

Each of the tested BCAAs was initially dissolved in Tris-HCl buffer to give initial stock solutions. Then it was to give final concentrations of these acids range from 1.5 to 25 mM . Orlistat was used as positive control modulating the conversion of para-nitrophenolbutyrate into chromogenic para-nitrophenol (colorimetrically determined). Which was procured from Santa Cruz (USA). Subsequent determinations were undertaken for the tested Ile, Leu, Val or BCAAs combination, in comparison to negative control (untreated sample) evaluations, the concentration required for PL 50% inhibition was calculated (IC50 value).

**Statistical analysis**

The results were presented as means± standard deviation (SD) of 3-4 independent experiments. GraphPad Prism ANOVA followed by Dunnett's post-hoc test was used to measure the Statistical differences between sample, a p-value of less than 0.05 was statistically significant. P-values of less than 0.001 were a highly significant.
**Results**

*Effect of BCAA on pancreatic acute glucose stimulated Insulin secretion (GSIS)*

The pancreatic MIN6 β-cell line was the cellular model to study the insulinotropic as well as the proliferation activity of BCAA (Ile, Leu, Val and BCAA combination). MIN6 is well-characterized as a higher insulin secreting cell compared to other β cell lines. Submaximal stimulatory glucose concentration (5.6mM) used as culture incubations. L-Ala at (10mM) was used as positive control [10]. Fig 1 shows that L-Ala at 10mM has significantly and dose-dependently potentiated GSIS in MIN6 β-cell line compared to untreated sample (p<0.001 vs. 5.6mM glucose alone) without compromising cell viability Table 1. Likewise, Leu (15 and 25mM) significantly and dose-dependently enhanced the pancreatic insulin secretion (p<0.001 vs. both 5.6mM glucose and 10 mM L-Ala; Fig 1. Moreover, Ile and Val (8, 15 and 25mM) enhanced substantially the MIN6 insulin secretion (p<0.001 vs. both 5.6 mM glucose and 10 mM L-Ala), respectively. Impressively, the BCAA combination significantly enhanced the insulin secretion (4-15mM) (p<0.001 vs. 5.6mM glucose) and (p<0.01 vs. 10 mM L-Ala; Fig 1. In consistency with L-alanine, the insulin secretion under the influence of Leu (2, 4, 8, 15 and 25mM), Ile (15 and 25mM) and BCAAs combination (1, 2, 4 and 8mM) were completely stopped in the absence of Ca$^{2+}$ (p<0.001), (p<0.05) and (p<0.001), respectively, as compared to respective Ca$^{2+}$–supplemented wells Fig 1. This indicates the major role of extracellular Ca$^{2+}$ in stimulating the insulin output by influx through voltage gated Ca$^{2+}$ channels. However, the subsequent induction of insulin exocytosis by influx the extracellular Ca$^{2+}$ through voltage gated Ca$^{2+}$ channels was not needed for Val Fig 1. In cell viability testing; Ile, Leu and Val showed no significant differences in the pancreatic beta cells at all concentrations compared to the positive control and negative control, in contrast to the BCAA combination at 15mM and 25mM Table 1.

![Fig 1. Modulatory effect A- Leu (mM) B- Ile (mM) C- Val (mM) and D- BCAA combinations (mM) on augmentation of pancreatic GSIS following acute 1h-treatment as evaluated by insulin ELISA. The result shows the insulin secretion percentage after acute exposure to 5.6 mM glucose with or without 2.5mM of calcium under different concentration of BCAAs. The results are expressed as means ± SD of three independent replicates. *: p<0.05, **: p<0.01, ***: p<0.001 compared to respective 10mM L-Ala, Δ: p<0.05, ΔΔ: p<0.01, ΔΔΔ: p<0.001 compared to respective 5.6mM glucose (negative control). Y: p<0.05, YY: p<0.01, YYY: p<0.001 compared to respective Ca$^{2+}$ free treatment condition in presence of 2.5 mM Ca$^{2+}$.](image-url)
Table 1. Modulatory effect of the tested Ile, Leu, Val and BCAAs combinations and the L-Ala as positive control after 1 h of incubation with 5.6 mM of glucose with or without 2.5mM of calcium on the viability of pancreatic β cells of MIN6 in post respective treatment measured by SRB dye.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>viability of MIN6 under glucose 5.6 mM</th>
<th>viability of MIN6 under glucose 5.6 mM without 2.5mM of calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nM) 1</td>
<td>2</td>
</tr>
<tr>
<td>Ile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCAA combinations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Ala 10mM</td>
<td>96.5±0.9</td>
<td></td>
</tr>
</tbody>
</table>

The result demonstrates the percentage of MIN6 viability after acute exposure to 5.6mM glucose with or without 2.5mM of calcium under different concentration of BCAA (mM). The result is expressed as means ± SD of three independent replicates.

**Cell proliferation and viability assays**

**Fig. 2** show that GLP-1 at 500nM can enhance a high β cell proliferation significantly (p<0.001 vs. spontaneous control). More effectively Ile at 15 and 25mM, Leu at 4, 8, 15, and 25mM, Val at 15, 25mM and BCAA combinations at, 2, 4 and 8 mM, augmented BrdU incorporation (p<0.001 vs. both, spontaneous control and GLP-1) **Fig. 2**.
Regarding the viability; Ile at doses 1-25 mM preserved cell integrity. Similarly, Leu and Val at doses 1-25 mM had no viability-compromising properties. However, the integrity of the cell affected by BCAA combination at 15mM and 25mM Table 2.

Table 2. Modulatory effect of Ile, Leu, Val and BCAAs combinations, and the 500nM GLP as positive control on the viability of pancreatic β cells of MIN6 in 48 h post seeding measured by SRB dye.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MIN6 viability (as % control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mM)</td>
</tr>
<tr>
<td>Ile</td>
<td>97.6±0.1 96.7±1.2 96.9±0.2 109±0.3 125±0.5 137.6±1.4</td>
</tr>
<tr>
<td>Leu</td>
<td>96.7±0.3 96.8±0.3 118.3±1 125.8±0.3 129.3±0.5 133.5±3.1</td>
</tr>
<tr>
<td>Val</td>
<td>97.6±0.7 97.2±5.6 96.5±1.1 96.7±0.6 119.6±1.4 131.9±5.4</td>
</tr>
<tr>
<td>BCAAs combinations</td>
<td>99.4±0.2 135.2±3.1 129.3±2.5 117.5±0.9 74.9±1.8 71.3±0.3</td>
</tr>
<tr>
<td>GLP-1(500nM)</td>
<td>99.7±0.8</td>
</tr>
</tbody>
</table>

The result represents percentage of the viable MIN6 cell line after chronic exposure to different concentration of A-BCAAs (mM). The expressed as means of the three measurements ± SD of three independent replicates.

**Insulinotropic effects of BCAAs in glucotoxicity, lipotoxicity and glucolipotoxicity**

To study the effect of BCAAs on pancreatic β cells under chronic hyperglycemia and hyperlipidemia; mice insulinoma MIN6 β-cell line were cultured underglucotoxicity, lipotoxicity and glucolipotoxicity conditions. Fig. 3 shows that L-Ala at 10 mM has significantly (p<0.01) potentiated the acute insulin secretion in MIN6 β-cell line under glucotoxicity condition and significantly (p<0.05) under lipotoxicity and glucolipotoxicity conditions as compared to control wells.

Similarly, Ile significantly enhanced GSIS (p<0.01 vs. untreated controls) under glucotoxicity, lipotoxicity and glucolipotoxicity conditions. However, Leu and BCAAs combination enhanced MIN6 GSIS (p<0.05 vs. both untreated sample), under glucotoxicity and lipotoxicity (but not glucolipotoxicity) Fig. 3.

**Inhibition of enzymatic starch digestion under the influence of BCAAs**

It is well documented that α-amylase and α-glucosidase inhibitors may reduce postprandial plasma glucose level via retarding the liberation of D-glucose from oligosaccharides, disaccharides and dietary complex carbohydrates thereby delaying glucose absorption. Releasing glucose from culinary polymeric cornstarch was inhibited substantially by acarbose (0.1 mg/mL)
(p<0.001 vs. control) in Fig. 4. Furthermore, Fig. 4 demonstrates that Ile (2.5, 5 and 10mM) and Leu (5 and 10mM) have dose-dependently inhibitions of releasing glucose from starch (p<0.001 vs. control) with respective IC50 values of Ile; 162.6±24.4 mM and Leu; 47.7±7.1mM Table 3. Fig. 4 illustrates the highly-pronounced decreases in enzymatic culinary corn starch hydrolysis by concentration gradient (1–10 mM) of BCAAs combination (p<0.001 vs. control) with an IC50 value 6±0.9 mM Table 3. Surprisingly, Arg (though unbranched) could be equally effective in exerting the dual amylase/glucoamylase inhibition dose in dependently Fig 4.

### Table 3. In vitro enzymatic starch digestion IC50 values for Ile, Leu, Val, BCAAs combinations and acarbose used as reference drug.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ile</th>
<th>Leu</th>
<th>Val</th>
<th>BCAA combination</th>
<th>Reference drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic Starch Digestion IC50 (mM)</td>
<td>162.6±24.4</td>
<td>47.7±7.1</td>
<td>NI</td>
<td>6±0.9</td>
<td>Acarbose 0.2± 0.02 µg /mL</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD of three independent replicates. NI: corresponds to non-inhibitory over the concentration range (0.5 – 10mM)

**Fig 4.** In vitro inhibitory effects of A- Leu, B- Ile and C- BCAA combinations (0.5-10mM) and 100µg/mL Acarbose on enzymatic starch digestion. The results represent the released glucose at mM after exposure the α-amylase and α-glucosidase enzymes to different concentration Ile. The results are expressed as mean ± SD of three independent replicates. The results were analyzed using one-way ANOVA. *: p<0.05, *: p<0.01, ***: p<0.001 compared to control.

**Inhibitory effects of BCAA on PL activity**

PL was inhibited substantially by Orlistat's PL-IC50 of 0.114 ± 4.0 µg /mL, equivalent to 0.2 ± 0.0 µM, [13]. The tested amino acids including Ile, Val and BCAAs combinations were recognized for their inhibition to PL activity with their respective IC50 values represented in Table 4 and in Fig. 5. However
unlike Orlistat’s, none of the investigated amino acids could be identified as equally potent.

Table 4. In vitro enzymatic PL IC50 values for Ile, Val, BCAAs combinations, and Orlistat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ile</th>
<th>Leu</th>
<th>Val</th>
<th>BCAA combination</th>
<th>Reference drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic Triacylglycerol Lipase IC50 (mM)</td>
<td>109.5±13.4</td>
<td>NI</td>
<td>248±5.6</td>
<td>22.9±0.6</td>
<td>Orlistat 0.114 ± 0.01 µg/mL</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD of three independent replicates. NI: corresponds to non-inhibitory over the concentration range (1.5-25 mM).

Fig. 5. In vitro inhibitory effects of Ile, Val and BCAA combinations and orlistat on PL activity. Results are expressed as mean ± SD of three independent replicates.

Discussion

Generally, Insulin released from β-cells in order to maintain homeostasis of glucose as a response to high level of glucose. So abnormal secretion of insulin due to the defect in β-cells may contribute to development of diabetes mellitus [14], the secretory function of β-cell regulated via modification of ion channel electrical activity. After glucose enter β-cell the rate of ATP production will increased, followed by depolarization of β-cell membrane as a response for closing the ATP gated K+ channels. Eventually the voltage gated Ca2+ channels will open and the insulin degranulation will initiate as a result for Ca2+ influx through these channels [15,16]. In this study we clearly showed the glucose-dependent insulinotropic under the influence of Ile, Leu, and BCAA combination in MIN6 cell line. The insulin secretory mechanisms under the effect of these acids was regulated by the adding Ca2+, this emphasize the relationships of depolarizing β-cells as well as stimulating Ca2+ influx, with the pivotal function of BCAA in increasing an endogenously regulated insulin secretion, which may suggest a prospective therapeutic approach for the treatment of T2DM.

As a result to increase insulin resistance β-cells keep dividing in order to maintain glucose level with in normal range and to prevent the development of T2DM. Several studies indicated that regulation of the function and mass of β cells associated with AKT/PKB signaling pathway [17,18], which is responsible for regulating the activation of cell growth, synthesis glycogen and protein, glucose
transport, as well as cell differentiation and survival [19]. In diabetes the low expression level of cyclin-dependent kinases (Cdk4), which is responsible for activation level of cyclins D, lead to growth retardation and reduction of the β-cell mass, however up regulation level of Cdk4 by activation of AKT/PKB pathway causes abnormal β-cell proliferation [20]. Consistent with our experiment β-cells showed that replication can be among the mechanisms for the maintenance of β-cell secretory function, which was further confirmed by our BrdU experiments. Furthermore, we suggest that the activation of Mammalian target of rapamycin (mTOR) by BCAA can initiate a cascade reaction leading eventually to increase the mass of β-cell. However, higher concentrations of these acids cause hyperactivity in the mTOR, leading to negatively regulates IRS1 by phosphorylation at serine residue, therefore inactivating the AKT/PKB pathway leading to inhibiting the effect of insulin on the proliferation of β-cell [21].

The pathophysiology of T2DM results in chronic hyperglycemia and hyperlipidemia, which are the major factors for progressive deterioration of pancreatic β-cell function [22]. The pancreatic MIN6 cell line were incubated in high concentration of glucose and fatty acids, to study the influence of the BCAA under the effect of glucotoxicity, lipotoxicity and gluco-lipotoxicity [12]. The results show significant restoration of the secretory function under the influence of BCAA. Multiple mechanisms have been suggested through which chronic hyperglycemia and hyperlipidemia may impair beta cell function [22] including: prolonged exposure to increased glucose concentrations causing gradual loss of insulin gene expression [23], the high demand on ER for the synthesis of proinsulin leading to cellular stress [24], increases the metabolic flux into the mitochondria and accumulation of intermediates of lipid metabolism induce excessive generation of ROS which leads to chronic oxidative stress and eventually to inflammatory response that trigger apoptosis by initiating pro inflammatory signal [25]. Therefore, we suggest that these acids could have antioxidant ability or not, which could be the reason for restoring the secretory function of the pancreatic β-cell under the effect of glucotoxicity and lipotoxicity. It was reported that addition of an antioxidant compound, or a reactive oxygen scavenger can protect the islet cells from oxidative stress causing the reverse of defects of glucose-stimulated stress in type 2 diabetic islets, and it was found that insulin secretion function from diabetic islets did not improve in the absence of Glutathione, which indicate that the decline in beta cell function is associated with increase of oxidative stress. Several studies have also demonstrated that amino acids could inhibit the NF-κB signal transduction pathways, contributing to the attenuation of inflammation and oxidation stress [26-28]. However, the present results are the first to show that a BCAA have effects on pancreatic MIN6 cell line regarding insulin secretion and proliferation.

One of the alternative therapeutic approaches against T2DM is decreasing lipid and glucose absorption by reducing the activity of α-amylase and lipase in the intestine [29]. In this study the results showed that BCAAs inhibit the activity of α-amylase and α-glucosidase, which decrease the releasing of D-glucose from starch, indicating that administration of these acids to diabetic subjects have inhibitory effect on the key enzymes related to hyperglycemia. We also showed that PL, which is a key enzyme of hydrolyzes non-absorbable triglycerides into simple glycerol and fatty acids, inactivated under the effect of BCAAs which can considered as...
valuable therapeutic compound to treat diet-induced obesity [30].

Conclusions

This study has showed that BCAAs could significantly improve glucose and lipid homeostasis by enhancing insulin secretion, and delaying carbohydrates and lipid absorption and digestion by inhibited amylase and lipase enzyme, respectively. Furthermore, BCAAs have insulinotropic efficacy in MIN6 β-cell and can protect the islet cells from oxidative stress causing the reverse of defects in type 2 diabetic islets. Therefore, BCAA represent potentially useful protection from diabetes. Furthermore, we recommended in the future studies: to investigate the effect of BCAAs on oxidation stress enzymes including catalase, glutathione peroxidase and xanthine oxidase regarding their activity and gene expression, which has been strongly linked to the development and progression of diabetic neuropathy.

Acknowledgements. The authors wish to thank the School of Pharmacy at the University of Jordan for input on the study.

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Romanian Journal of Diabetes Nutrition & Metabolic Diseases / Vol. 26 / no. 2 / 2019


