CHAPTER 2

Hydrolysed casein decreases postprandial glucose concentrations in T2DM patients, irrespective of leucine content

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ABSTRACT

BACKGROUND Lifestyle modifications, including diet, are important in the prevention and management of type 2 diabetes mellitus (T2DM). However, limited information is available on the effects of single doses of meal replacements, particularly with regard to their effect on postprandial glucose. Therefore a study was performed comparing the effects of a single meal replacement in T2DM patients on postprandial serum glucose, insulin and glucagon.

METHODS This randomized, double-blind, partial cross-over study was performed in 36 T2DM patients who continued their oral anti-diabetic medication. Each patient received three out of four treatments separated by 7 days. The treatments were a proprietary casein hydrolysate (insuVida™) alone or with additional leucine, unhydrolysed casein or placebo. Blood sampling was done for 4 hrs. Treatments were compared using repeated measures ANOVA. Results are given as an estimate of the difference (%) for the 4 hrs epoch.

RESULTS Glucose concentrations were lowered by 4.7% by insuVida™ and insuVida™ plus added leucine compared to placebo (95%CI: -1.6% to -7.7%) while the effect of unhydrolysed casein was -1.7% (-4.8% to 1.5%). Addition of leucine to insuVida™ induced the greatest increase in insulin (51.8%; 41.1% to 63.4%). All three treatments increased glucagon concentrations by 14% (8% to 20%) compared to placebo.

CONCLUSIONS A single dose of insuVida™ with or without addition of leucine significantly lowered plasma glucose compared to placebo and intact casein in T2DM patients. This is most likely due to an insulinotropic effect of insuVida™. The data suggest that this type of intervention may be a viable treatment strategy in T2DM.

INTRODUCTION

Lifestyle modifications such as diet, have beneficial effects on glucose metabolism in type 2 diabetes mellitus (T2DM) and are considered the first line of action to halt or delay further progression of the disease [1]. The development of ingredients for a functional food could be an interesting addition to the current dietary recommendations. Patients with T2DM can be characterised in part by impaired insulin response upon intake of carbohydrates such as glucose. Therefore, insulin secretagogues such as sulfonylurea derivatives are an important class of antidiabetic medication [2]. Amino acids are known to have insulinotropic properties as well [3-5]. The possibility that substances other than glucose could stimulate insulin secretion was first reported by Cochrane *et al*. in 1956, who showed that casein ingestion could induce acute hypoglycemia in children with familial idiopathic hypoglycemia [6]. Subsequently, many studies have demonstrated that the combined intake of carbohydrate and protein induced a higher insulin response than the intake of carbohydrate alone [7;8], both in healthy subjects [9-11] and in T2DM patients [12-17].

Specific amino acids, such as leucine, have been shown to further augment the insulin response of protein co-ingestion in healthy subjects [11;18] and type 2 diabetes mellitus patients [17;19]. Leucine stimulates insulin secretion in pancreatic ß-cells by increasing mitochondrial metabolism by activation of glutamate dehydrogenase (GDH) and increasing ATP (adenosinetrifosfaat) production by transamination of leucine [20;21]. Although the hypoglycaemic effect of proteins was first noted in the 1960s [7;8], a practical application of this effect has only been explored more thoroughly during the last ten years. One of these applications is the use of a protein hydrolysate as a meal replacement. Research with the proprietary casein hydrolysate insuVida™ (insV, formerly known as InsuVital™) has shown that ingestion of this product with carbohydrate augments the insulin response and enhances glucose disposal in patients with long-standing T2DM [12;15;19], which makes it an attractive food-based intervention. However, some of these studies were performed using a fairly high amount of protein (25-85 gram protein). Hence, these interventions were associated with a high protein load which would be undesirable in a subset of patients with diabetes mellitus who may have an impaired renal function. The addition of a protein hydrolysate could be used in a functional or clinical food for diabetic subjects if lower amounts of protein would also produce significant benefits in terms of glucose management. However, efficacy at a lower dose has not previously been demonstrated and therefore further research is needed to compare intact protein versus protein hydrolysate and on the additional effect of leucine on glucose homeostasis. Therefore, in a randomized, double-blind, placebo-controlled, partial cross-over study, we compared the insulinotropic, glycaemic and glucagonaemic effects of a low dose of a proprietary casein hydrolysate, with and without added leucine, against its native intact protein in individuals with stable-treated type 2 diabetes. The patients continued their medication in order to demonstrate whether the effects of the hydrolysate could add beneficial effects on top of medication.

METHODS

The study was conducted in accordance with the Declaration of Helsinki and Guideline for Good Clinical Practice. The protocol of this study was approved by the Medical Ethics Committee of the Leiden University Medical Center (LUMC). After informed consent was obtained, all patients were screened approximately three weeks before start of the study to assess eligibility. Screening consisted of a medical examination, ECG, vital signs, standard urine analysis and, hematology, virology, chemistry and HbA1c laboratory tests.

SUBJECTS

The study was performed in 36 patients of either gender, between 50 and 70 years of age and with an established diagnosis of T2DM as evidenced by the use of oral antidiabetic medication for at least one year and a fasting plasma glucose concentration of ≥ 7.0 mmol/L at screening after abstaining from oral antidiabetic medication for at least two days. Exclusion criteria were use of insulin, body mass index (BMI) > 35 kg/m², (recent) pregnancy, significant history of ischemic heart disease or congestive heart failure, uncontrolled hypertension, severe diabetic retinopathy, impaired liver function or renal function. After inclusion, patients were randomized according to the procedure described by Wakeling and MacFie [22]. A partial cross-over design was chosen in which each participant received three out of the four possible treatments with wash-out periods of 7 days. This was chosen to decrease the burden for the patient without under-powering the study.

TREATMENTS

The interventions were offered as a breakfast meal replacement shake that was freshly prepared prior to use by dissolving a sachet with 300 mL cold water. The treatments were administered between 9.00 and 9.30 AM. The energy content of the shakes was standardized and comparable with a normal breakfast. No information on the composition of the contents in the sachets was visible for either the participants or the research staff. Three out of the following four treatments were given (Table 1):

- Placebo
- Unhydrolysed casein
- insuVida™ (insV)
- insuVida™+leucine (insV+leu)

For each of the treatments the amount of carbohydrate, fat and vitamins was identical, except for the addition of 0.25 mg chromium picolinate and minerals for the insV treatments. All beverages were uniformly lemon flavoured. insuVida™ (DSM Food Specialties, Delft, The Netherlands) is a casein hydrolysate which is obtained by enzymatic hydrolysis of sodium caseinate. The amount and composition of the amino acids of both the intact and the hydrolyzed protein is the same (15 gram).

PROTOCOL

Patients continued to take their antidiabetic medication after screening. They maintained their normal dietary and physical activity patterns throughout the entire experimental period, but refrained from heavy physical labour and exercise training for at least 2 days before each study day. The enrolled patients participated on three study days in total with a wash-out periods of one week between two occasions.

In the evening prior to each study day, the patients took a standardized meal with a total energy content of 2676 kJ. The macro-nutrient energy distribution was 55 energy % carbohydrate, 28 energy % fat, and 17 energy % protein. This was followed by an overnight fast of at least 12 hours. During study days, patients remained fasted and refrained from xanthine-containing products as long as measurements continued. Lunch was offered after completion of the last measurement.

BLOOD SAMPLING AND ANALYSIS

Blood samples were drawn by sampling from an intravenous cannula inserted into a forearm vein. Blood was collected at -5, 0, 10, 20, 30, 40, 50, 60, 90, 120, 150, 180, 210 and 240 minutes after treatment ingestion for glucose, insulin and glucagon. Blood samples for insulin and glucose were collected in non-additive tubes. The samples were allowed to clot for 30-45 minutes and subsequently centrifuged at 4°C for 10 minutes at 2000 g. Samples were stored below -20°C until analysis. Blood samples for glucagon were collected in pre-chilled EDTA tubes. The samples were put on ice and within one minute 50 µl, 500 KIE Trasylol (Bayer, Germany) was added, centrifuged at 4°C for 10 minutes at 2000 g and within 30 minutes after collecting stored at a minimum of -20°C until analysis.

Glucose and insulin concentrations were measured in an automated assay using a fully automated Immulite analyzer (Immulite 2500 Analyzer Assay, EURO/DPC, UK). Glucagon was measured using a radioimmunoassay (RIA, Linco Research Inc., St Charles, MO, USA). The assays were performed at the Central Laboratories for Clinical Chemistry of LUMC. All assays were validated to manufacturer standards prior to study sample analysis. All samples from the same subject were processed within the same batch.

POWER CALCULATION

As data from former studies did not yield enough information for a cross-over power calculation, a conservative power calculation with relatively high standard deviation and a power of 90% was conducted. This resulted in a necessary effect size of 21.7 mmol/L*4hrs , assuming a standard deviation of differences of 39.0, using a paired t-test with a 0.05 two-sided significance level for 36 subjects.

STATISTICAL ANALYSIS

All data were entered into the analyses. Pharmacodynamic endpoints (postprandial serum glucose, insulin and glucagon levels) were analyzed separately by mixed model analysis of variance with treatment, occasion visit, time and treatment by time as fixed effects, with subject, subject by time and subject

by treatment as random effect (subjects received three of four possible treatments), and with the (average) baseline value as covariate. Variables were analyzed after log transformation and least square means (LSM) estimates were calculated within the model. All data handling and statistics were performed using SAS for Windows version 9.1.2 (SAS Institute, Inc, Cary, NC, USA).

RESULTS

SUBJECT CHARACTERISTICS

Sixty subjects were screened for the study. Twenty-four subjects were excluded from participation because they did not meet the inclusion criteria. Thirty-six patients entered the study; 27 males and 9 females. Demographics of the patients are shown in Table 2. Eleven of 15 patients on monotherapy for T2DM used a biguanide drug.

Twelve patients used a combination of a biguanide and sulfonylurea derivative, and six a biguanide and a thiazolidinedione drug. Three patients were on triple therapy. The majority of the patients received multi-modality treatment because of the increased cardiovascular risk associated with T2DM; 19 patients used statins, 23 antihypertensive medication and 10 patients received anticoagulant or antiplatelet therapy. Occasional use of analgesics or antacids was also reported, but none of concomitantly used drugs were considered to interfere with the objectives of the study (Table 3).

TOLERABILITY

No significant adverse events (AE) were recorded during this study. The most frequently occurring AE was gastrointestinal-related and consisted of abdominal cramps with or without diarrhea (n=7) or constipation (n=2) and nausea (n=1). The AEs were mild, transient and did not need intervention. No significant differences between treatments were found for the occurrence of AEs. All 36 subjects finished the study and attended all three occasions.

HORMONES AND GLUCOSE

The effect on average serum glucose concentration of insV treatment with or without added leucine was indistinguishable, and both interventions resulted in 4.7% lower glucose concentrations compared to placebo (p=0.0036, 95% CI: -7.7% to -1.6% for both, Figure 1, Table 4).

There was no difference in postprandial glucose concentrations between placebo and the treatment with unhydrolysed protein (95%CI from -4.8% to 1.5%). The difference in effect on average serum glucose concentrations between the unhydrolysed protein and both insV treatments was 3%. Glucose declined to baseline at approximately 3 hours and was below baseline for the remainder of the observation period.

Average insulin concentrations increased rapidly after intake of all protein containing meal replacements (Figure 2). The strongest insulin increase compared to placebo occurred after insV with added leucine (Table 4: 51.8%; 95% CI: 41.1 to 63.4%, expressed as estimate of the difference over the 4 hour observation period). The increase in insulin after insV treatment without leucine (26.1%) was significantly less than the increase in insulin after unhydrolysed protein (36.0%).

Average glucagon attained peak concentrations at approximately 30 minutes and declined below baseline approximately 1 to 1.5 hours after treatment intake (Figure 3). The increase of glucagon after placebo was minimal. The increase in glucagon after the treatment with unhydrolysed protein and both insV treatments compared to placebo was similar and amounted to approximately 14% (95% CI: 7.5% to 20.2%, Figure 3).

DISCUSSION

Postprandial glucose concentration is increasingly seen as an important determinant for management of HbA1c concentrations [1;23]. A reduction in postprandial glucose of 0.8 mmol/L (about 10%) (due to diet and lifestyle intervention) is related to a reduction in T2DM incidence of 58% [24]. The diabetes prevention program research group showed that lifestyle intervention was significantly more effective than metformin (reduced incidence of 58% vs 31%) [25]. It is reasonable to assume that the combination of an energy-balanced diet and protein hydrolysates may have clinical relevance in opposing the development of insulin resistance by attenuating the postprandial rise in blood glucose concentration.

The aim of this study was to compare the effects of a single, low dose of hydrolysed protein, with or without added leucine, and of unhydrolysed protein on blood concentrations of glucose, insulin and glucagon in T2DM patients. The present data confirm results from earlier studies [12;15;19] that protein hydrolysates can lower postprandial glucose concentrations when taken along with a carbohydrate load. This study also shows that this effect is still present at much lower doses than employed previously. Many studies employed doses of around 0.3-0.4 g/kg hydrolysate [12;15;19;26] which is considerably higher than the 15 g protein (approximately 0.2 g/kg body weight) used in the current study. Furthermore, the effect of the tested hydrolysate on postprandial glucose does not depend on its leucine content, since treatment with either insV or insV+leu reduced postprandial glucose to a similar extend, even though insV+leu induced a larger insulin response. Both insV and

insV+leu outcompeted unhydrolysed protein in terms of glucose lowering. All three active treatments induced a relatively short lasting increase in glucagon concentrations.

There is ample evidence that certain proteins, protein hydrolysates and amino acids stimulate the release of insulin. Indeed, consumption of a combination of proteins and carbohydrates causes a more pronounced insulin response than ingestion of carbohydrates alone [7]. Previous research with insV in patients with T2DM has shown that co-ingestion of this product with carbohydrate augments the insulin response and enhances glucose disposal [12;15;19]. It should be noted that protein hydrolysate was administered on top of the carbohydrate shake that was provided in the placebo group, which induced a higher caloric load in the treatment groups. The fact that the hydrolysates lowered glycemic response even in the face of a higher caloric load further stresses the glucose lowering potential of the interventions.In this study the effect of additional leucine in hydrolysated casein was evaluated in view of contradictory previous findings [15;27]. The data indicate that this mixture induced the largest increase in insulin concentrations (Figure 2). However, the postprandial glucose responses following treatment with insV+leu were similar to treatment with insV without additional leucine. These findings are in line with an earlier postprandial study [15] with the same product. It is unclear why insV+leu induced a greater insulin response, but no additional lowering of plasma glucose, compared to insV. Glucagon responses were measured to assess whether the glucose response to the leucine-induced insulin increase would be offset by increased glucagon concentrations. This appeared not to be the case, since the glucagon responses to insV and insV+leu were similar (Figure 3).

INSUVIDA™ VERSUS UNHYDROLYSED PROTEIN

insV tended to lower glycemic responses compared to intact casein and this is significant over the 0-2 hr period (estimated of the difference -4.3%; 95%CI: -1.1 to -7.4%, Figure 1). These data support the notion that for postprandial glucose management, the added value of a casein hydrolysate as compared to intact casein is especially pronounced in the early post-prandial phase. This is probably due to a difference in rate of appearance of amino acids in the peripheral circulation between whole casein protein and the casein hydrolysate [28]. Casein protein and their respective peptide hydrolysates were emptied from the stomach at similar rates, but the speed of intestinal amino acid absorption was slower for the casein protein solution. Although a minute amount of chromium (0.25 mg) was present in the insV treatments, it is unlikely that this contributed to the difference between the hydrolysate and the unhydrolysed protein as none of the well-designed studies have ever demonstrated any effect of chromium on (post-prandial) glucose regulation in humans [29, 30].

In view of the size of the insulin response to protein-rich treatment, the postprandial glucose lowering effect is moderate [12]. It is well known that glucagon and insulin have opposite effects on (postprandial) glucose concentrations and are closely interlinked. Therefore, it is more important to consider the ratio of insulin to glucagon in evaluating a clinical situation rather than considering the concentrations of either hormone alone.

Plasma glucagon concentrations peaked before a decrease in plasma glucose occurred; respectively at 30 min and 60 min (Figure 1 and 3). Therefore, other factors than glucose concentrations must have been influencing this glucagon response. Proteins and amino acids stimulate both α- and ß cells in the pancreas, thus stimulating both the glucagon and the insulin release [9, 10, 31, 32]. Moreover, ß cells appear to be more responsive to protein than α cells [31]. It has been shown that glucagon release depends on the protein-to-carbohydrate ratio of the meal [33-35]; a higher ratio is associated with an increased glucagon release. Calbet *et al*. found that the glucagon response to protein feeding was linearly related to the plasma amino acid concentration [9]. Thus, lower protein loads would be more appropriate to induce higher Insulin/Glucagon (I/G) ratio, while by increasing the protein load mainly glucagon responses will be affected resulting in lower I/G ratios [31]. Therefore, it appears that the secretion of pancreatic glucagon during protein intake in association with insulin secretion, serves to limit the decline of glucose concentration. This may explain that hypoglycemia has never been observed in this or the previous studies with this hydrolysate.

A single dose of a casein hydrolysate with and without the addition of leucine enhanced the carbohydrate-induced insulin response in T2DM patients, resulting in significantly lower plasma glucose concentrations compared to placebo and intact casein. In addition, the secretion of pancreatic glucagon was increased during all three active treatments associated with increased insulin secretion. This mechanism may have limited the decline of plasma glucose concentrations and may be responsible for the absence of hypoglycemia. Further research is required to determine the long-term clinical benefit of a low dose of insuVida™, with and without additional leucine.

TABLE 1 Composition of individual treatments

	Placebo	Unhydrolysed protein	insuVida™ g/serve	insuVida™ & leucine
Protein: insuVida™ casein hydrolysate	-	-	17.61**	17.61**
Protein: Sodium caseinate (Saneigien)	-	15.0	-	-
L-leucine	-	-	-	5.00
Maltodextrin: Maldex G 120	17.00	17.00	17.00	17.00
Dextrose monohydrate (Meritose 200)	18.47	18.47	18.47	18.47
Vegetable oil (rapeseed)	5.00	5.00	5.00	5.00
Additives (specified below)	8.94	8.94	8.94*	8.94*

*Additives: Vitamin+Mineral premix: 2.85 gram, Gum (Xanthan and Guar) 0.48 and 0.12 gram, Sucralose 0.036 gram, Flavour lemon 0.3 gram, Lecithin: 0.15 gram and * Chromium 0.00025 gram. ** Net protein weight: 15 gram.*

TABLE2 Subject demographics

Gender	9 Females / 27 Males
Age (yrs)	61.5 ± 5.1
BMI (kg/m²)	28.1 ± 3.6
Diagnosis of T2DM (yrs)	7 ± 5
HbA1c (%)*	6.8 ± 0.9
Fasting glucose (mmol/L)	9.6 ± 2.3
Total cholesterol (mmol/L)	4.67 ± 1.02
Triglycerides (mmol/L)	1.57 ± 0.70

** HbA1c glycated hemoglobin*

TABLE 3 Concomitant medication

Oral antidiabetics	Monotreatment	n=15
	Dual treatment	n=18
	Triple treatment	n= 3
Cardiovascular	Lipid Lowering	n=19
	Blood pressure lowering	n=23
	Anticoagulant/antiplatelet	n=10
Central nervous System	Analgesic / Occasional sedative	n=6
GI tract	Antacid	n=4
Endocrine	Thyroid hormone	n=2
Urogenital tract	Prostate hypertrophy	n=2
Pulmonary tract	Anti-allergic	n=1
	Anti-asthma	n=1
Miscellaneous	Eye drops / vitamins / Acetylcysteine / Allopurinol	n=5

TABLE 4 Summary of profile analysis results

Estimate of the difference (%) of postprandial serum glucose levels. Insulin and glucagon levels from 0-4 hours after treatment ingestion with 95% confidence interval and p-value.

	Protein vs. Placebo	insV vs. Placebo	insV + leu vs. Placebo	insV vs. Protein	insV vs. insV + leu	insV + leu vs. Protein
Serum glucose 0-4 hrs (mmol/L)	-1.7% (-4.8%, 1.5%) p=0.2781	-4.7% (-7.7%, -1.6%) p=0.0036	-4.7% (-7.7%, -1.6%) p=0.0038	-3.0% (-6.1%, 0.1%) p=0.0578	0.0% (-3.1%, 3.3%) p=0.9810	-3.0% (-6.1%, 0.1%) p=0.0610
Insulin 0-4hrs (mU/L)	36.0% (26.4%, 46.3%) p=<.0001	26.1% (17.1%, 35.7%) p=<.0001	51.8% (41.1%, 63.4%) p=<.0001	-7.3% (-13.8%, -0.2%) p=0.0430	20.4% (11.9%, 29.6%) p=<.0001	11.7% (3.8%, 20.1%) p=0.0037
Glucagon 0-4 hrs (ng/L)	13.8% (7.7%, 20.2%) p=<.0001	13.5% (7.5%, 19.8%) p=<.0001	13.8% (7.8%, 20.2%) p=<.0001	-0.3% (-5.6%, 5.3%) p=0.9120	0.3% (-5.0%, 5.9%) p=0.9142	-0.0% (-5.3%, 5.6%) p=0.9973

FIGURE 1 LSMS Serum glucose for each treatment

Dot: placebo, Triangle: unhydrolysed casein, Square: insV, Circle: insV+leucine. The figure represents the time course of the least square mean of serum glucose (with 95% CI error bars). Significant differences as compared with placebo are indicated for insV and insV+leucine (p<0.003).

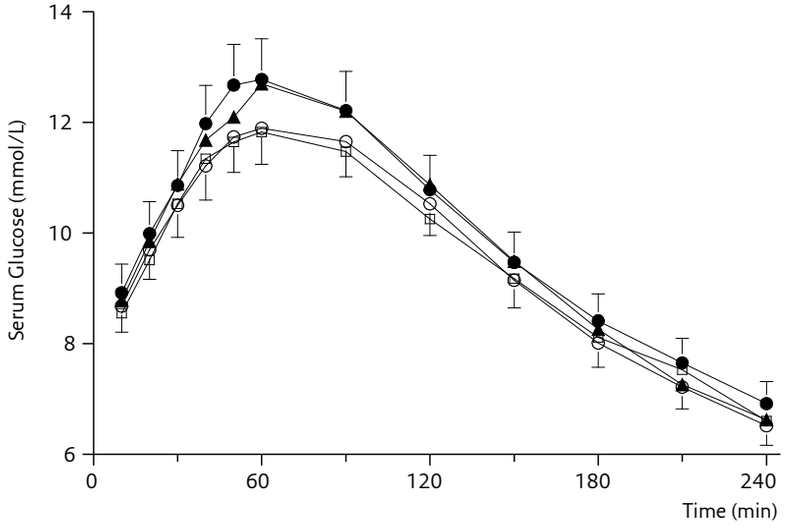


FIGURE 2 LSMS Insulin for each treatment

Dot: placebo, Triangle: unhydrolysed casein, Square: insV; Circle: insV + leucine. The figure represents the time course of the least square mean of insulin (with 95% CI error bars). Significant differences as compared with placebo are indicated for all three treatments (p<0.0001).

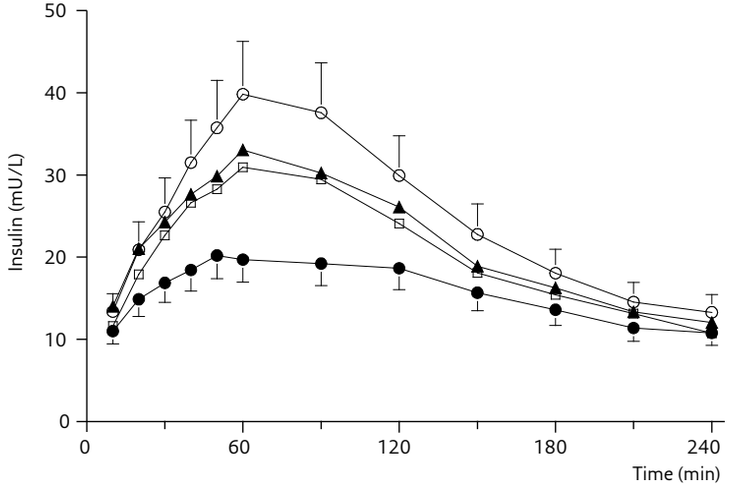
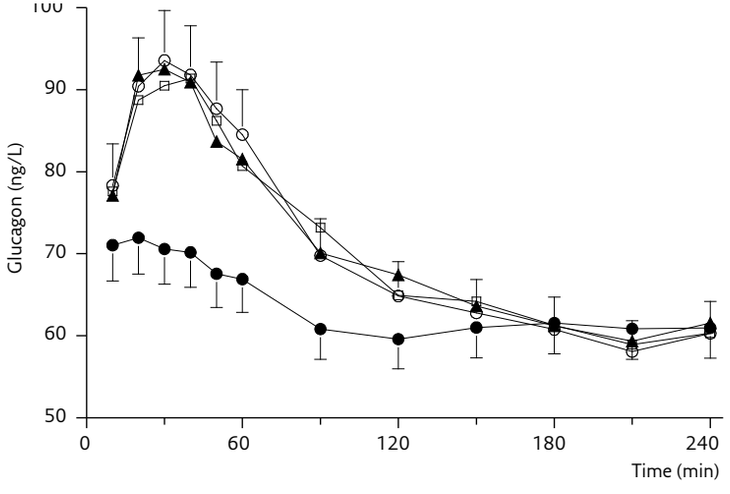


FIGURE 3 LSMS Glucagon for each treatment

Dot: placebo, Triangle: unhydrolysed casein, Square: insV, Circle: insV+leucine. The figure represents the time course of the least square mean of glucagon (with 95% CI error bars). Significant differences as compared with placebo are indicated for all three treatments (p<0.0001).



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