What goes up must come down: glucose variability and glucose control in diabetes and critical illness
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Chapter 5

A randomised clinical trial comparing the effect of basal insulin and inhaled mealtime insulin on glucose variability and oxidative stress

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Abstract

**Aim:** To assess the effect of three times daily mealtime inhaled insulin therapy compared with once daily basal insulin glargine therapy on 72-hr glucose profiles, glucose variability and oxidative stress in type 2 diabetes patients.

**Methods:** In an inpatient crossover study, 40 subjects with type 2 diabetes were randomised to receive 9 days of inhaled insulin three times daily before meals or 9 days of glargine administered in the morning before breakfast in a randomised order. During the last 72 hrs in each phase, glucose was measured with continuous glucose monitoring. Activation of oxidative stress was measured by determining the $15(S)$-8-iso-PGF$_{2\alpha}$ secretion in 24-hr urine samples.

**Results:** Inhaled insulin improved overall and postprandial glucose control significantly better than insulin glargine ($P<0.0001$). There was a trend towards a greater reduction in glucose variability (8-9%) in the inhaled group ($P = 0.1430$ and $P = 0.3298$ for mean amplitude of glycaemic excursions (MAGE) and mean of daily differences, respectively). Oxidative stress, estimated by determining the urinary isoprostane excretion ($15(S)$-8-iso-PGF$_{2\alpha}$), was equally reduced from baseline by both treatments. No correlation was found between glucose variability and oxidative stress in both groups.

**Conclusions:** This study showed a mealtime insulin approach to improve glycaemic control more than a basal insulin approach. These findings indicate also that lowering glucose using insulin treatment lowers oxidative stress over time, at least for the study period of 9 days, in type 2 diabetes patients. Contrary to earlier data, we found no correlation between glucose variability (MAGE) and oxidative stress ($15(S)$-8-iso-PGF$_{2\alpha}$) in this study.
Effect of basal and inhaled mealtime insulin on glucose variability and oxidative stress

Introduction

It has been suggested that glucose variability, considered in combination with haemoglobin A1c (HbA1c), is a more reliable indicator of blood glucose control and the risk for long-term complications than HbA1c alone\(^1^\)–\(^3\), and if so, this has important bearings for the people with diabetes. Still the impact of glucose variability on macrovascular complications and its effect in type 2 diabetic patients has not been firmly established.

In an attempt to provide evidence for this hypothesis, Monnier et al.\(^4\) showed a strong correlation between glucose variability (expressed as mean amplitude of glycaemic excursions [MAGE]) and oxidative stress (measured as 8-isoprostane excretion) in type 2 diabetes patients, suggesting a relationship between glucose variability and diabetic complications. However, in type 1 diabetes patients, a disease with more pronounced glucose variability, we could not find a correlation between MAGE and urinary 8-isoprostane excretion\(^5\). To further test this hypothesis, an intervention study aiming at reducing variability specifically in the intervention group without affecting mean glycaemia more than in the control group is rational. Hirsch and Brownlee\(^1\) suggested to perform a randomised controlled trial comparing a regimen of mealtime and basal insulin with a regimen of basal insulin alone in newly diagnosed type 2 diabetes patients.

The present study compared a mealtime insulin regimen (inhaled insulin) with a basal insulin regimen (insulin glargine) in a crossover design in type 2 diabetes patients failing on oral medication. In a study by Bretzel et al.\(^6\), using a similar design with a rapid-acting analogue rather than inhaled insulin, indeed overall glycaemia was reduced by both treatments to a similar extent, while postprandial values were lower in the mealtime insulin group and fasting glucose was lower in the basal insulin group. Thus, glucose variability was by necessity more reduced in the mealtime insulin group, making it possible to study glucose variability in both groups independent from the influence of overall glycaemia. We report the first intervention study specifically targeting glucose variability in insulin-treated type 2 diabetes patients.

Methods

Subjects

The study population consisted of patients with type 2 diabetes mellitus poorly controlled on a combination of two oral agents. Patients had to be at least 18 years old with type 2 diabetes diagnosed 6 months prior to study entry and currently be treated on a stable dose of at least 2 oral hypoglycaemic agents for at least 1 month prior study entry.
with or without adjunct subcutaneous insulin. Patients had to have a screening HbA1c ≥6.5% and ≤10.5% and a BMI ≤44 kg/m². A complete list of in- and exclusion criteria can be found in the paper describing the glycaemic outcome of this study. (Hompesch et al.) The study was carried out in accordance with the principles of the Declaration of Helsinki and of Good Clinical Practice. All local regulatory requirements were followed. Before entering the study, patients gave written informed consent after a detailed oral and written explanation of the study procedures.

**Study design and procedure**
This was a prospective, open-label, randomised, two-period crossover trial. The study consisted of a screening visit and two 9-day inpatient periods separated by a 7- to 10-day washout period. Subjects agreed to participate in this study by signing an informed consent. Subjects were randomised 1:1 to inhaled insulin (Exubera®, Pfizer, New York, NY, USA) three times daily before meals or glargine (Lantus®, sanofi-aventis, Paris, France) administered in the morning of the second day before breakfast, then switched treatments for the second phase (Figure 1). Patients who had existing subcutaneous insulin regimens discontinued those therapies prior to each inpatient stay but resumed them during washout. All patients did continue their pre-study oral diabetic treatments throughout the study.

![Figure 1 Study design](image)

On the fifth day of each inpatient stay, vascular access for frequent venous blood sampling was secured and subjects were subsequently connected to an automated glucose monitoring system (CGMS® system gold, Medtronic MiniMed, Northridge, CA, USA)
inserted in the abdominal wall for 72 hrs. Average interstitial glucose (IG) levels calibrated to blood glucose were stored at 5-min intervals. The CGMS device was calibrated according to the manufacturer’s instructions. For analysis, only data generated in the 72 hrs between 06:00 hrs on day 6 and 06:00 hrs on day 9 for each phase were used. During both inpatient CGMS glucose profile periods, urine and blood samples were collected. Urinary 8-iso-PGF$_{2\alpha}$ was sampled from 24-hr urine collection on days 1 and 8 of each inpatient period.

**Laboratory measurements**

Activation of oxidative stress was estimated by determining the urinary isoprostane excretion (15(S)-8-iso-PGF$_{2\alpha}$), using high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). Urine samples were collected and stored without additives as 7 ml aliquots at -80 °C. Initial creatinine concentrations were established by colourimetric Jaffé assay. Two millilitres of sample were mixed with $^2$H$_4$-labelled 15(S)-8-iso-PGF$_{2\alpha}$ as internal standard and applied to an 8-iso-PGF$_{2\alpha}$ immunoaffinity column (Cayman Chemical Company, Ann Arbor, MI, USA). After washing, the extract was eluted, evaporated to dryness (60 °C, N$_2$) and reconstituted in 200 μl 0.05 mol/l formic acid-ethanol (75:25, v/v); 50 μl was injected on the HPLC-MS/MS system. Chromatographic separation was achieved on a modular HPLC system (Surveyor, Thermo Finnigan, San Jose, CA, USA) consisting of a cooled autosampler ($T = 12^\circ$C), a low-flow quaternary MS pump and analytical HPLC column: Alltima C8, 2.1 x 150 mm, 5 μm (Alltech, Lexinton, KY, USA). Samples were eluted with a flow rate of 200 μl/min and a programmed linear gradient between A (0.01% HCOOH in H$_2$O; v/v) and B (CH$_3$CN): from $t = 0$ min 45% A, 55% B towards $t = 3$ min 30% A, 70% B towards $t = 3.1$ min 100% A until $t = 6$ min. MS/MS analyses were performed on a TSQ Quantum AM (Thermo Finnigan) operated in the negative ion electrospray ionisation mode. The surface-induced dissociation was set at 2 V; spray voltage was 3500 V and the capillary temperature was 400°C. In the MS/MS experiments argon was used as collision gas at a pressure of 0.2 Pa; collision energy was 26 eV for the optimised transitions: m/z 353.24 $\rightarrow$ m/z 193.10. The interassay (n = 5) and intraassay (average of 5 days, n = 3) variability allowed for determination at physiological concentrations with a coefficient of variation of <7%.

**Assessment of glycaemic variability**

*Interday glycaemic variability*

The day-to-day variation of the glucose pattern was calculated with the mean of the daily differences (MODD), which is defined as the mean of the absolute differences between glucose values on day 2 and the corresponding values on day 1, at the same time $^8$.$^9$.

*Intraday glycaemic variability*

This was calculated by the MAGE $^10$. The MAGE over 24 hrs is the mean of the absolute differences between peak and nadir values over 24 hrs, with peaks (nadir) defined as
glucose values preceded and followed by an increase (decrease) and decrease (increase), respectively, in excess of at least 1 SD of the mean glucose. If a decrease of more than 1 SD was the first excursion, only peak-to-nadir excursions (>1 SD) were included in the calculation of the MAGE and vice versa.

**Statistical analysis**

No sample size calculations were performed since this was an exploratory study. Pharmacodynamic endpoints, including the mean 72-hr glucose profiles; the area under the IG concentration-time curve over 72 hrs (IG-AUC_{0-72h}); the 3-day mean IG-AUC_{0-24h}; postmeal IG-AUC_{0-4h} for breakfast, lunch, and dinner; the 3-day mean IG-AUC_{0-24h} at night time; the 3-day mean maximum glucose concentration after breakfast IG-C_{max}; and the time to IG-C_{max} (IG-t_{max}) were derived from the two glucose exposure profiles measured with the CGMS. Glycaemic exposure measures (AUCs) were calculated using the trapezoidal rule. To evaluate the variability of glucose exposure, the following parameters were calculated: the SD of the mean 72-hr glucose concentration, the MAGE over 72 hrs^{10}, and the MODD of paired glucose levels over 72 hrs using standard statistics. The differences in 15(S)-8-iso-PGF\_2α levels are analyzed using a prespecified mixed effects model procedure in SAS (version 8.02, SAS Institute, Cary, NC, USA) with treatment, sequence and period as fixed effects and patient within sequence considered a random effect. Normality assumptions were checked as necessary, with log transformation to improve this. Correlation was calculated and univariate regression analysis was performed to evaluate the relation between the excretion rate of 15(S)-8-iso-PGF\_2α and the markers of glycaemic variability. Locally weighted polynomial regression (LOESS) curves were added in Figure 2. Statistical significance for all results is expressed through 95% CIs, whereby a finding is deemed significant when neither side of the confidence interval crosses 1.0 (or 100%).

**Results**

A total of 40 patients with type 2 diabetes (male, 29; age, 57 ± 10 y; BMI, 31.9 ± 4.6 kg/m\(^2\); HbA1C, 7.9 ± 1.0%; mean prebreakfast capillary glucose at randomization 8.1 ± 2.2 mmol/l; type 2 diabetes duration 10.2 years [range 0.8 – 27.0]) were enrolled. Diabetes was treated with oral antidiabetic medication (39 patients received metformin, 28 sulfonylurea, 11 rosiglitazone, and 5 pioglitazone); five patients were also treated with glargine. During the study, two patients did not complete the two treatment sequences. Two patients discontinued during glargine treatment, one during the first period (and consequently did not receive inhaled insulin in the second period), and one during the second period (having completed treatment on inhaled insulin in the first period).
Effect of basal and inhaled mealtime insulin on glucose variability and oxidative stress

The mealtime targeted approach with inhaled insulin improved overall and postprandial glucose control (expressed as total glycaemic exposure over 72 hrs; mean glucose concentration over the final 72-hr period; the 3-day mean glycaemic exposure; 4-hr postmeal glycaemic exposure after breakfast, lunch, dinner; and the 3-day mean maximum glucose levels after breakfast) significantly better than the basal insulin approach using glargine ($P < 0.0001$; Table 1).

As hypothesised, glucose variability was more reduced in the inhaled group compared to the glargine group, however not significantly (8-9% reduction; $P = 0.1430$, $P = 0.3298$ and $P = 0.1613$ for MAGE, MODD and SD respectively) (Table 1).

Oxidative stress, estimated by determining the urinary isoprostane excretion (15(S)-8-iso-PGF$_{2\alpha}$), was reduced by both treatments (Table 2). There was no evidence of a period effect, looking at the baseline 8-isoprostane values after the washout periods that were higher than after finishing treatment. There was a trend towards a somewhat greater reduction in oxidative stress in the glargine group compared to the inhaled group.

We found no correlation between oxidative stress (urinary isoprostane excretion) and glucose variability (expressed as MAGE and MODD) (Figure 2). The correlation coefficients ($r$) for the inhaled and glargine group were 0.18 and 0.28 for MAGE and 0.17 and 0.20 for MODD respectively.
### Table 1 Glycaemic exposure and variability of glycaemic exposure

| Glycaemic exposure (mean ± SD) | Inhaled (n = 38) | Glargine (n = 38) | Inh/Gla+ (95% CI) | P-value* |
|-------------------------------|-----------------|-----------------|-------------------|---------|
| Mean 72 hrs (mmol/l)          | 5.3 ± 0.6       | 6.0 ± 1.1       | 88 (84-93)        | <0.0001 |
| AUC72h (mmol/l·hr)            | 380.3 ± 45.3    | 426.3 ± 89.2    | 89 (84-93)        | <0.0001 |
| AUC0-24h 3-day mean (mmol/l·hr)| 126.5 ± 15.1    | 142.5 ± 27.9    | 89 (84-93)        | <0.0001 |
| AUC0-4h post breakfast (mmol/l·hr) | 25.9 ± 4.8    | 27.7 ± 5.2      | 93 (87-99)        | 0.0318  |
| AUC0-4h post lunch (mmol/l·hr) | 19.0 ± 4.0      | 24.6 ± 5.7      | 78 (72-84)        | <0.0001 |
| AUC0-4h post dinner (mmol/l·hr)| 20.5 ± 4.0      | 26.3 ± 5.7      | 78 (72-84)        | <0.0001 |
| AUC0-6h night time (mmol/l·hr) | 29.6 ± 4.6      | 31.0 ± 6.4      | 96 (91-103)       | 0.2345  |
| Cmax post breakfast (mmol/l·hr) | 8.3 ± 1.8       | 8.8 ± 1.7       | 94 (87-100)       | 0.0572  |
| tmax post breakfast (hrs)     | 1.5 ± 0.6       | 1.8 ± 0.7       | 83 (71-97)        | 0.0180  |

| Variability of glycaemic exposure (mean ± SD) | Inhaled (n = 38) | Glargine (n = 38) | P-value* |
|-----------------------------------------------|-----------------|-----------------|---------|
| SD 72 hrs (mmol/l)                            | 1.5 ± 0.6       | 1.6 ± 0.6       | 92 (81-104) | 0.1613  |
| MAGE 72 hrs (mmol/l)                          | 3.5 ± 1.4       | 3.7 ± 1.3       | 91 (79-104) | 0.1430  |
| MODD 72 hrs (mmol/l)                          | 1.4 ± 0.5       | 1.5 ± 0.6       | 93 (81-107) | 0.3289  |

*Treatment/reference ratio (%) of the estimates of the geometric means from the mixed model fitted to the natural-log transformed endpoint data. 

P-value is for the estimated treatment effect between inhaled and glargine from the mixed model fitted to the natural-log transformed endpoint data. AUC, area under concentration-time curve; Cmax, maximum concentration; tmax, time to maximum concentration; SD, standard deviation; MAGE, mean amplitude of glycaemic excursions; MODD, mean of daily differences.

### Table 2 15(S)-8-iso-PGF$_2$α of creatinine

| Baseline | End of treatment | ∆* | 99% CI |
|----------|-----------------|----|-------|
| Inhaled  | 0.06 (0.03)     | 0.05 (0.03) | 0.01 (0.02) | -0.020, 0.000 |
| Glargine | 0.06 (0.03)     | 0.05 (0.02) | 0.02 (0.02) | -0.030, -0.010 |

15(S)-8-iso-PGF$_2$α of creatinine is expressed in nmol/mmol creatinine. ∆ represents the difference in 15(S)-8-iso-PGF$_2$α concentrations from baseline to end of treatment. 99% confidence interval is chosen because of the small design of the study.
Discussion

The present study assessed the effect of three times daily mealtime insulin (inhaled) therapy compared to once daily basal insulin (glargine) therapy on 72-hr glucose profiles, glucose variability and oxidative stress in type 2 diabetes patients.

At least two important conclusions can be drawn. First, this study reveals that glucose lowering using insulin treatment lowers oxidative stress (Table 2). In both groups, there was a significant decline in 8-isoprostane production during treatment. As there was no indication of a period effect, a time effect can be excluded. Lowering oxidative stress as a result of lowering glycaemia was so far only reported as a momentary effect. Ceriello et al. described earlier a significant reduction of postprandial oxidative stress in type 1 diabetes patients when reducing postprandial glucose excursions with pramlintide, an amylin analogue. They examined nitrotyrosine, oxidised LDL and total radical-trapping antioxidant parameter during a 4-hr postprandial period. They found a correlation between the extent of postprandial glycaemia and the oxidative stress. Our data strengthen their findings and extend the hypothesis of an oxidative stress lowering effect by lowering glucose to a period of 8 days.

Second, we found no correlation between oxidative stress, measured as 8-iso-PGF$_{2\alpha}$ in urine, and glucose variability, defined as MAGE (Table 1, Figure 2). This is in accordance with an earlier study in type 1 diabetes patients. On the other hand, Monnier et al. reported a strong relationship between glucose variability and oxidative stress in type 2 diabetes patients. An explanation for the lack of correlation between glucose variability and oxidative stress can be a methodological issue. We used next to immunoaffinity isolation highly selective HPLC tandem MS for detection instead of the less specific enzyme immunoassay to quantify 8-isoprostanes: HPLC-MS/MS is not hampered by cross-reactivity of structurally (un)related components of 8-iso-PGF$_{2\alpha}$, whereas the immunoassay is more susceptible to interference, as acknowledged in the earlier Monnier report. An alternative possibility is that a relationship between glucose variability and oxidative stress only exists in non-insulin treated type 2 diabetes patients. Recently, Ceriello reported a clamp study suggesting that oscillating glucose can have more deleterious effects than constant high glucose on endothelial function and oxidative stress. However, in this study only two glucose excursions were elicited, what in our opinion shows that a second repetitive episode of acute hyperglycaemia elicits more oxidative stress than the first, which is somewhat different than glucose variability over the whole day. Looking at the consequence of oxidative stress, that is vascular complications, Gordin et al. detected no correlation between glucose variability (expressed as MAGE) and arterial stiffness, considered an early sign of macrovascular complications, in type 1 diabetes patients.
Overall glycaemic control was significantly better with a mealtime insulin approach using inhalation insulin compared to a once daily approach using glargine. Literature is not in agreement on this subject, varying from no difference between both regimens to significantly better glycaemic control (expressed as decrease in HbA1c) when using a mealtime approach in patients with type 2 diabetes starting with insulin therapy. The overall better glycaemic control in the mealtime insulin group confounded the comparison in oxidative stress between both groups, as lowering glycaemia lowers oxidative stress.

In our study, glucose variability in the mealtime insulin group was somewhat lower than in the once daily insulin group (Table 1) albeit not significantly. We think that the non-significance is likely mainly explained by the small and therefore underpowered study group. As postprandial hyperglycaemia accounts for the major part of glucose variability, certainly in type 2 diabetes patients who experience few hypoglycaemic episodes, one would expect glucose variability to be smaller with a mealtime insulin approach. Glucose variability in the inhaled group was enlarged by the twofold higher incidence of mild and moderate hypoglycaemia in the inhaled group. It is therefore likely that the variability in the mealtime insulin group would have been lower if overall glycaemic control would have been the same. It is also possible that glucose variability in the basal insulin group is lower than expected. This could be explained by the residual beta-cell function of the patients treated until recently with oral medication, mitigating the postprandial glucose to rise even without mealtime insulin administration.

We have no clear explanation why oxidative stress seemed somewhat more lowered in the glargine group. From the literature one would have expected lower oxidative stress in the mealtime insulin group, resulting either from better overall glycaemic control in the inhaled group or from reduced variability. Possibly, the lower oxidative stress level in the basal insulin group is a spurious finding. Again, a larger trial would be needed to answer this question more definitively.

In conclusion, this study shows that lowering glucose lowers oxidative stress in type 2 diabetes patients not only as a momentary effect, extending the existing data of Ceriello to a longer period. Second, we found no correlation between glucose variability and oxidative stress in insulin-treated type 2 diabetes patients. Finally, a non-significant almost 10% decline in glucose variability did not result in lower oxidative stress in insulin-treated type 2 diabetes patients.

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