Diabetes Publish Ahead of Print, published online February 25, 2008

Oscillating glucose is more deleterious on endothelial function and oxidative stress than mean glucose in normals and type 2 diabetic patients.

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Running Title: Oscillating glycemia and endothelial dysfunction

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Received for publication 16 January 2008 and accepted in revised form 19 February 2008.

ABSTRACT

Background: Much attention has been recently paid to the possibility that oscillating glucose may super impose to HbA1c levels in determining the risk for cardiovascular diabetic complications.

Methods and Results: A euinsulinemic hyperglycemic clamp at 5, 10 and 15 mmol/liter glucose was given in increasing steps; as a single "spike;" or oscillating between basal and high levels over 24 hr in normal subjects and type 2 diabetic patients; flow mediated dilation, a marker of endothelial function; and plasma 3-nitrotyrosine and 24-hour urinary excretion rates of free 8-iso prostaglandin F2alpha, two markers of oxidative stress, were measured over 48 hr post-clamp. Glucose at two different levels (10 and 15 mmol/liter) resulted in a concentration-dependent fasting blood glucose-independent induction of both endothelial dysfunction and oxidative stress in both normal and type 2 diabetic patients. Oscillating glucose between 5 and 15 mmol/liter every 6 hr for 24 hr resulted in further significant increases in endothelial dysfunction and oxidative stress as compared to either continuous 10 or 15 mmol/liter glucose and that concomitant vitamin C infusion can reverse this impairment.

Conclusions: These data suggest that oscillating glucose can have more deleterious effects than constant high glucose on endothelial function and oxidative stress, two key players in favouring cardiovascular complications in diabetes.

KEYWORDS. Oscillating glucose, oxidative stress, endothelial dysfunction

A strong relationship between the mean levels of glycemia, measured as HbA1c, and diabetic complications (1-2), including cardiovascular complications (3) has been demonstrated. However, what is still unclear is whether glycemic instability may confer additional risk to the development of complications over that predicted by the mean glucose value alone. It is therefore unknown if two individuals with the same mean blood glucose, but extremes of glucose variability, might have the same or different level of risk for complications.

In favour of this association is that in a 1995 report of the DCCT, the risk of retinopathy progression associated with a given level of mean HbA1c differed significantly between intensively and conventionally treated patients (4). It was suggested that this may be a consequence of larger glycemic excursions in the conventional group (4).

However, about cardiovascular complications, it has been reported that fasting plasma glucose instability is a predictor of cardiovascular-related mortality in elderly patients with NIDDM and it has been suggested that glucose stability might be a goal in the management of these patients (5).

A related issue is the evidence that postprandial glycemia is a stronger risk marker for macrovascular complications than fasting glucose (6).

Endothelial dysfunction is predictive of a future cardiovascular event (7) and evidence indicates that hyperglycemia induces endothelial dysfunction through an oxidative stress (8), which is a key player in favouring diabetic complications (9).

The aim of this study is to verify the effect of glucose variability on endothelial function and the possible involvement of oxidative stress in the phenomenon.

MATERIALS AND METHODS

Subjects. 27 type 2 diabetic patients and 22 healthy subjects were recruited (Table I). The diabetic patients had newly diagnosed (within 6 months) type 2 diabetes mellitus and were on diet alone and had no evidence of any cardiovascular complications.

The protocol of the study was approved by the ethics committee of our institution. All subjects gave informed consent before being tested.

Methodology. As described in the Study Design, the subjects of this study underwent periods of hyperglycemia and periods of normoglycemia. Below the techniques used to attain these conditions are described.

In order to maintain euinsulinemia, endogenous insulin secretion was inhibited during all the experiments using somatostatin (Sandostatin, Novartis Pharma, Basel, Switzerland) (10). Somatostatin was infused in two phases: 1) a bolus dose of 25 μg over 1 min given 5 min before the start of the experiment and 2) as a continuous maintenance dose of 1.0 μg/min (10).

Hyperglycemic euinsulinemic clamp. The hyperglycemic euinsulinemic clamp was a modification of the method used by Del Prato et al (10).

Normalization of glycemia. Insulin and/or 5% glucose to keep blood glucose levels between 4 and 6 mmol/liter were started (11). Blood glucose levels were determined every 5 minutes with adjustment of the intravenous insulin infusion, until steady-state glucose levels were between 4 and 6 mmol/liter . At the steady state, venous glucose samples were drawn every 30 minutes.

Control study with somatostastin. 5 normal subjects and 5 diabetic patients underwent to a control study with 3 h somatostatin infusion alone and endothelial function as well as nitrotyrosine plasma levels were evaluated. No change in these parameters was found.

Study Design. *Protocols.* Two different clamp protocols were planned and the diabetic patients as well as the normal subjects participating to each protocol were matched for age, sex, BMI, and for fasting glycemia and Hba1c in the case of diabetes.

A: **Oscillating high and normal glucose:** this protocol was designed to explore the direct effect of glucose variability compared to stable hyperglycemia on endothelial function and oxidative stress production.
In 12 normal subjects and in 15 diabetic patients, in randomized order, for 24 hours, glycemia:
1. was increased at 15 mmol/liter every six hours and normalized for the further six hours;
2. maintained at 15 mmol/liter (peak value);
3. maintained at 10 mmol/liter (mean glycemia value per 24 hours of experiment 1);
and FMD and nitrotyrosine measured every six hours during the experiments and 24 hours after the experiment end.
B: Oscillating glucose plus vitamin C: this protocol was designed to explore the effect of oxidative stress on the endothelial dysfunction induced by oscillating glucose.
In 10 normal subjects and in 12 diabetic patients, in randomized order, for 24 hours, glycemia: was increased at 15 mmol/liter every six hours and normalized for the further six hours with
or without vitamin C infusion [3 mg/min] (11), and FMD and nitrotyrosine measured every six hours during the experiments and 24 hours after the experiment end.
In both the protocols 24-hour urinary excretion rate of free 8-iso prostaglandin F2alpha (8-iso PGF2alpha) was also measured.
Biochemical Measurements. Plasma glucose ,cholesterol, triglycerides, HDL-C and HbA1c were measured by routine laboratory methods. LDL-C was calculated after lipoprotein separation (12).
NT was measured by ELISA (13). Free 8-iso prostaglandin F2alpha plasma levels were measured by a commercially available kit (Cayman Chemical**,** Ann Arbor, Michigan, U.S.A.).
Endothelial Function. Endothelial function was evaluated using flow-mediated vasodilatation (FMD) of the brachial artery. The validity of this method has been confirmed in previous studies (14-15). At the end of each test, the subjects lay quietly for 15 minutes. Then, sublingual nitroglycerine (0.3 mg) was administered and 3 minutes later the last measurements were performed. Response to nitroglycerine was used as a measure of endothelium-independent vasodilatation. In this study we were able to confirm, as previously reported, that inter-observer variability for repeated measurements of resting arterial diameter was 0.04±0.02 mm, while the intra-observer variability for repeated measurements of resting arterial diameter was 0.02±0.02 mm (8).
Statistical Analysis. *The number of subjects to include in each protocol was selected according to the previous available literature (16-19)*
The Kolmogorov-Smirnov algorithm was used to determine whether each variable had a normal distribution. Comparisons of baseline data among the groups were performed using unpaired Student's *t* test. The changes in variables during the tests were assessed by two-way ANOVA with repeated measures. If differences reached statistical significance, post hoc analyses with two-tailed paired *t* test was used to assess differences at individual time periods in the study, using Bonferroni correction for multiple comparisons. Statistical significance was defined as $p<0.05$.
Data are reported as Mean±SEM.

RESULTS

The baseline differences between diabetic and normal subjects are reported in the Table I.
Protocol A: oscillating high and normal glucose as compared to high constant glucose
Clamping glycemia at 10 and 15 mmol/liter produced an impairment of endothelial function ($p< 0.01$ vs basal at any time) and an increase in nitrotyrosine generation ($p< 0.01$ vs basal at any time), in both normal and diabetic patients (Figures 1 and 2).
Normal Subjects: Endothelial dysfunction ($p< 0.01$ at any time) and nitrotyrosine levels ($p< 0.01$ at any time, except at 18h

p=NS) were significantly increased in response to 15 as compared to 10 mmol/liter glucose. Oscillation of glycemia from basal levels to 15 mmol/liter produced a significant endothelial dysfunction (at 6h p< 0.01 vs basal, at 18h p< 0.001 vs basal) and increase in nitrotyrosine levels (at 6h p< 0.01 vs basal, at 18h p< 0.001 vs basal). In oscillating glucose as compared to constant glucose at 15 mmol/liter, after the first 6 h of hyperglycemia, levels of endothelial dysfunction and nitrotyrosine were super imposable (P=NS). At18 h, however, after the second period of hyperglycemia, both endothelial dysfunction (p< 0.01) and nitrotyrosine levels (p< 0.01) were higher in the oscillating glucose. The effect of oscillating glucose as compared to 10 mmol/liter constant glucose was even more pronounced than the 15 mmol/liter results for both endothelial dysfunction and nitrotyrosine (6h and 18h; p< 0.01). During the oscillating period, even when glycemia was normal, neither endothelial function or nitrotyrosine returned to the basal levels. The data are reported in the figure 1.

Diabetic Patients: Endothelial dysfunction (p< 0.05 at any time) and nitrotyrosine levels (p< 0.01 at any time, except at 18h p=NS) were significantly increased in response to 15 as compared to 10 mmol/liter constant glucose in diabetic patients.

At 12 h, in oscillating situation, after the first period of hyperglycemia, both endothelial dysfunction and nitrotyrosine were super imposable to the levels observed at the same time point in response to 15 mmol/liter constant high glucose, and significantly different from those observed at 10 mmol/liter (p< 0.05 and p< 0.01, respectively) at 12 h. At 24 hours, however, endothelial dysfunction was significantly increased as compared to both 10 and 15 mmol/liter constant glucose groups (p< 0.01 vs both), while nitrotyrosine was significantly increased as compared to 10 mmol/liter (p< 0.001) but not as compared to 15 mmol/liter (p= NS). In diabetic subjects, as compared to both 10 and 15 mmol/liter constant glucose, normalizing glucose levels in the oscillating group resulted in improved endothelial dysfunction and nitrotyrosine levels. The data are reported in the figure 2.

The 24-hour urinary excretion rates of free 8-iso prostaglandin F2alpha was significantly higher in the oscillating glucose groups as compared to either constant high 15 mmol/ liter or 10 mmol/liter groups in both normal [342±52 vs 271±54 (p< 0.05), vs 230±35 (p< 0.01) pg/mg creatinine] and diabetic patients [536±51 vs 476±48 (p< 0.05), vs 432±47 (p< 0.01) pg/mg creatinine].

Protocol B: oscillating glucose plus vitamin C. In the normal subjects at 6 h and 18 h, both endothelial dysfunction (p< 0.01 and p< 0.001, respectively) and nitrotyrosine levels (p< 0.01 and p< 0.001, respectively) were significantly different in response to oscillating glucose as compared to basal time points. The values of both endothelial function and nitrotyorisine were at 18h significantly different from those at 6h (p< 0.01) in response to oscillating glucose. The infusion of vitamin C counterbalanced the effect of oscillating glucose (Figure 3).

In the diabetic patients at 12 h and 24 h, both endothelial dysfunction (p< 0.01 and p< 0.001, respectively) and nitrotyrosine levels (p< 0.01 and p< 0.001, respectively) were significantly different as compared to basal time points in response to oscillating glucose. The values of both endothelial function and nitrotyorisine were at 24h significantly different from those at 12h (p< 0.01) in response to oscillating glucose. The infusion of vitamin C counterbalanced the effect of oscillating glucose (Figure 4).

However, in normal subjects, vitamin C not only counterbalanced the effect of oscillating glucose, but almost normalized both endothelial function and nitrotyrosine levels (Figure 3). In diabetic patients, vitamin C, during high glucose periods, maintained endothelial function and nitrotyrosine at basal levels, however, during the periods of glucose normalization, vitamin C further improved, as compared to

baseline, both endothelial dysfunction and nitrotyrosine levels (Figure 4).

The 24-hour urinary excretion rates of free 8-iso prostaglandin F2alpha were significantly higher in the oscillating glucose group without vitamin C infusion in both normal (351±88 vs 160±55 pg/mg creatinine, $p< 0.001$) and diabetic patients (550±78 vs 320±78 pg/mg creatinine, $p< 0.01$).

Endothelium-independent vasodilation did not change during any of the tests (data not shown).

DISCUSSION

In this study we have been able to show that oscillating glucose, over a period of 24 hours, is more damaging to the endothelial function than stable constant high glucose. This is true not only when a subject is exposed to the same total amount of glucose for 24 hours (i.e., 10 mmol/liter clamp), but even when the total amount is higher (i.e., 15 mmol/liter clamp). Finally, data suggest that oxidative stress plays a key role in all of these phenomena.

Many reports have already shown that an acute increase of glycemia induces endothelial dysfunction in both normal and diabetic subjects (8, 16-19). However, in these experiments only the effect of a single increase, for few hours, of glycemia has been evaluated. For the first time, we can show that fluctuations of glycemia are always accompanied by an impact on endothelial function. This effect seems to be related to the level of glycemia reached. This is not surprising: a strong direct correlation has been already demonstrated between the endothelial function and the level of glycemia reached during an oral glucose tolerance test (OGTT) in normal subjects, in people with impaired glucose tolerance (IGT), and with overt diabetes (19) or when hyperglycemia is differently managed in the postprandial state (20-21).

Again for the first time, we are able to show in humans that the effect of oscillating glucose is worse than that of constant high glucose. This concept has been widely demonstrated in both cells and animals (22-25). Interestingly, in our study the effect is independent of total amount of glucose to which subjects are exposed. Similar results have already been obtained in vitro, in human renal cortical fibroblasts (26).

Even if oxidative stress generation appears to be the key player of all the phenomena observed in the study, the precise mechanism through which oscillating glucose may be worse than constant high glucose, remains not completely defined. Although further studies are certainly warranted, these would be quite difficult to accomplish in humans. Studies in cells and in animals, however, have shown that in oscillating glucose pathways involving PKC (22), NADPH (22), iNOS (27) or inflammatory markers are more activated in response to oscillating glucose as compared to constant high glucose (23-25). However, it has been also shown that the activation of many of these pathways is secondary to the generation of free radicals at the level of the mitochondria during high glucose overload (23, 28). As preliminarily reported, another possible explanation is that in oscillating glucose conditions the cells are not able in sufficiently increase their own intracellular antioxidant defenses (29), a condition which has been suggested to favour the development of diabetic complications (30-31). In this regard, a recent study showed that during acute hyperglycemia, in normal subjects, several genes involved in free radical detoxification were downregulated (32).

Even though there was an absence of significance in the nitrotyrosine levels at 24h between 15 mmol/liter constant glucose and oscillating glucose in diabetic patients, overall in this study, nitrotyrosine plasma levels were found to change according to the variations in glycemia in both normal and diabetic subjects, as has been previously published (8, 11, 33). Other factors, such as the activation of inflammation could be involved, however, we are able to confirm that glucose oscillation is accompanied by an increased excretion of free 8-iso

prostaglandin F2alpha in the 24 hours of each study (34). Even in the case of this marker, the oscillation of glucose is more harmful than constant high glucose. Finally, almost all the alterations induced by high glucose, either stable or oscillating are reversed by vitamin C.

An apparent limitation of this study is that a significant difference between oscillating glucose and the constant infusion of glucose can be observed only in the second pulse of oscillating glucose. In our opinion, this is exactly what we can expect from the design of the study. In fact, the first pulse of high glucose can simply show the effect of an acute increase of glycemia, as reported in many other studies. The effect of oscillation in glucose can be observed, by definition, only after the first pulse. More frequent pulses of glucose would better confirm the hypothesis, however, this kind of design could be very difficult to feasibly carry out.

The involvement of oxidative stress merits attention. In diabetes free radical over-production is not only involved in generating endothelial dysfunction (35) and linked to an increased risk of cardiovascular disease (36), but, specifically in this disease, it is strictly related to the development of the complications (9). More specifically, is certainly intriguing that nitrotyrosine (37) and free 8-iso prostaglandin F2alpha (38) are both independent risk factors for cardiovascular disease.

Other findings of this study may deserve attention. During the oscillating experiments, in normal subjects never did endothelial function and nitrotyrosine return to basal levels, while this happened in diabetic patients. Furthermore, vitamin C was able to normalize endothelial function and oxidative stress only in normal subjects, but not in diabetic subjects during the period of hyperglycemia, while when glycemia was normalized in diabetic patients, the simultaneous administration of vitamin C almost normalized both endothelial function and oxidative stress. A possible hypothetical explanation is that two pathways are simultaneously working: one due to the actual level of glycemia and another one to the long lasting damage induced in the endothelial cells by chronic hyperglycemia, possibly through non-enzymatic glycation of mitochondria (39). Although speculative, this explanation is consistent with a previous finding showing that only the simultaneous control of hyperglycemia together with vitamin C infusion can normalize endothelial function in diabetes (11).

Finally, a possible role for insulin, more than for the reduction of hyperglycemia, in determining the improvement of endothelial dysfunction might be supposed. However, even if recently, Ellger et al. have shown in an animal model that it is the reduction of glucose toxicity more than the action of insulin that improves endothelial function (40), the role of insulin as a protector against activation of oxidative stress cannot be completely excluded. Additional studies, such as the assessment of the relationship between markers of oxidative stress and insulin concentrations during an euglycemic hyperinsulinemic clamp, are needed.

Our results support the hypothesis that the oscillation of plasma glucose may impact on endothelial function and oxidative stress generation more than stable high glucose, two well recognized risk factors in diabetes for cardiovascular (35) complications. Even in light of these results, specific trials are needed for a final answer, we trust that the results of this study may contribute not only in giving a possible pathophysiological explanation to the phenomenon, but in raising the alert for a need to avoid glucose oscillation in clinical practice in order to prevent cardiovascular diabetic complications.

ACKNOWLEDGMENTS

The authors do not have conflicts of interest and all were allowed to have access to the data.

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	Controls (22)	Diabetics (27)
Sex	12 M 10F	14M 13F
Age	50.5±2.5	51.3±2.6
BMI Kg/m2	28.5±3.1	29.5±3.3
Fasting glucose mmol/l	4.5±0.3	7.8±2.2*
HbA1c %	4.8±0.2	7.7±0.3*
Resting systolic blood pressure mm Hg	117.3±5.5	123.4±6.4
Resting diastolic blood pressure mm Hg	77.5±2.2	80.2±3.6
Total cholesterol mmol/l	4.5±0.6	5.1±0.8
Triglycerides mmol/l	0.9±0.2	1.2±0.4
HDL-C mmol/l	1.4±0.2	1.2±0.3
LDL-C mmol/l	2.5±0.3	2.6±0.4
FMD %	11.7±0.7	5.9±0.6*
Nitrotyrosine µmol/l	0.24±0.5	0.52±0.3*

TABLE I: Baseline characteristics of the normal and diabetic subjects.
Data are expressed as mean±SEM *p< 0.001 vs controls

FIGURE LEGENDS

Figure 1. Hyperglycemic clamps in normal subjects. For 24 h, glycemia
1) was increased at 15 mmol/l every six h and normalized for the further six h(◊);
2) maintained at 15 mmol/l (●) (peak value);
3) maintained at 10 mmol/l (▲) (mean glycemia value/24 h) of experiment 1;
and FMD and nitrotyrosine measured during the experiments and after 24 h from the end.
Bars indicate SEM.

Figure 2. Hyperglycemic clamps in diabetic patients. For 24 h, glycemia
1) was increased at 15 mmol/l every six h and normalized for the further six h (◊);
2) maintained at 15 mmol/l (●) (peak value);
3) maintained at 10 mmol/l (mean glycemia value/24 h) of experiment 1 (▲);
and FMD and nitrotyrosine measured during the experiments and after 24 h from the end.
Bars indicate SEM.

Figure 3. Hyperglycemic clamps in normal subjects. For 24 h, glycemia was increased at 15 mmol/l every six h and normalized for the further six h without (◊) or with vitamin C infusion(●) 3 mg/m. FMD and nitrotyrosine measured during the experiments and after 24 h from the end.
Bars indicate SEM.

Figure 4. Hyperglycemic clamps in diabetic patients. For 24 h, glycemia was increased at 15 mmol/l every six h and normalized for the further six h without (◊) or with vitamin C infusion(●) 3 mg/m. FMD and nitrotyrosine measured during the experiments and after 24 h from the end.
Bars indicate SEM.

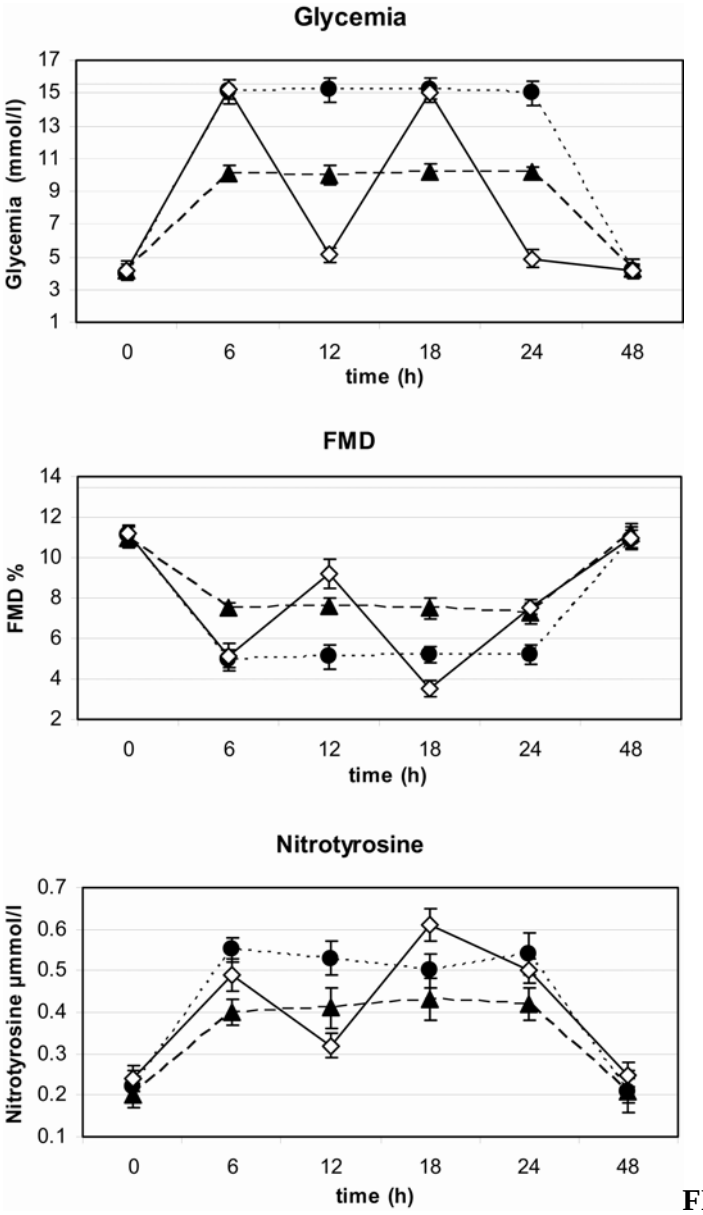
Glycemia
Glycemia (mmol/l)
17
15
13
11
9
7
5
3
1
0
6
12
18
24
48
time (h)
FMD
FMD %
14
12
10
8
6
4
2
0
6
12
18
24
48
time (h)
Nitrotyrosine
Nitrotyrosine µmmol/l
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
6
12
18
24
48
time (h)

FIGURE 1

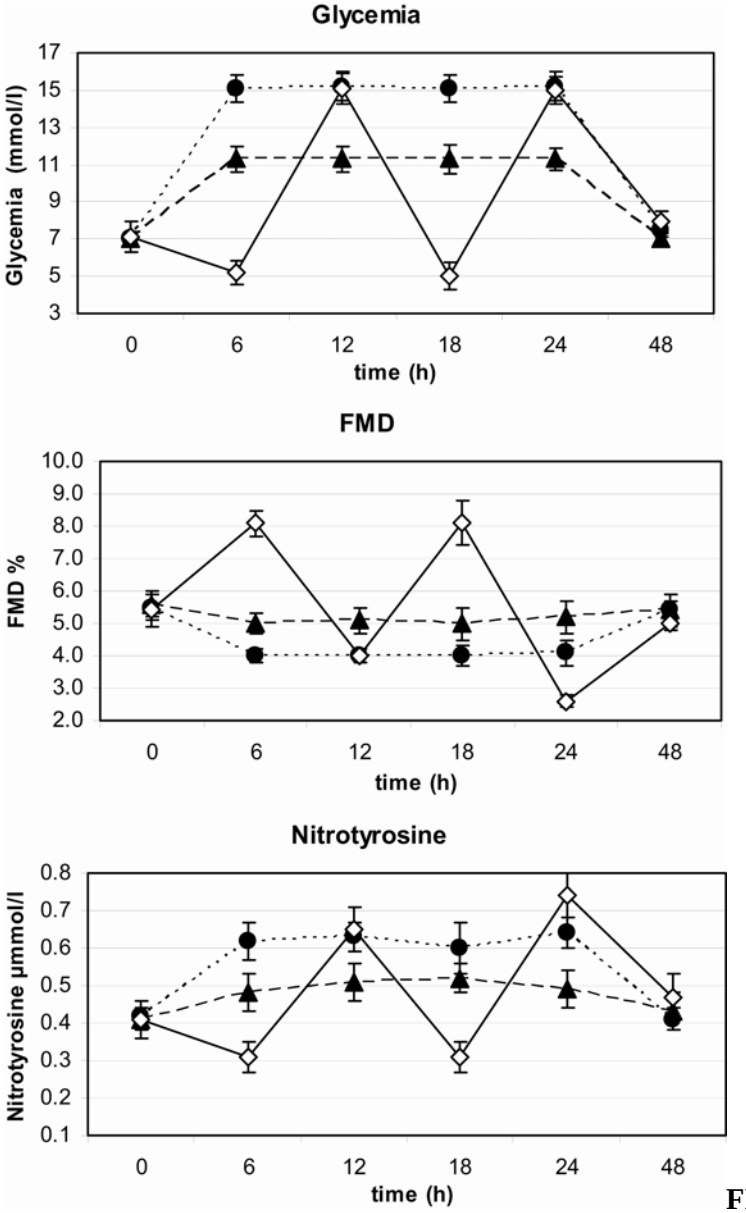
Glycemia
Glycemia (mmol/l)
17
15
13
11
9
7
5
3
0
6
12
18
24
48
time (h)
FMD
FMD %
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
0
6
12
18
24
48
time (h)
Nitrotyrosine
Nitrotyrosine µmmol/l
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
6
12
18
24
48
time (h)

FIGURE 2

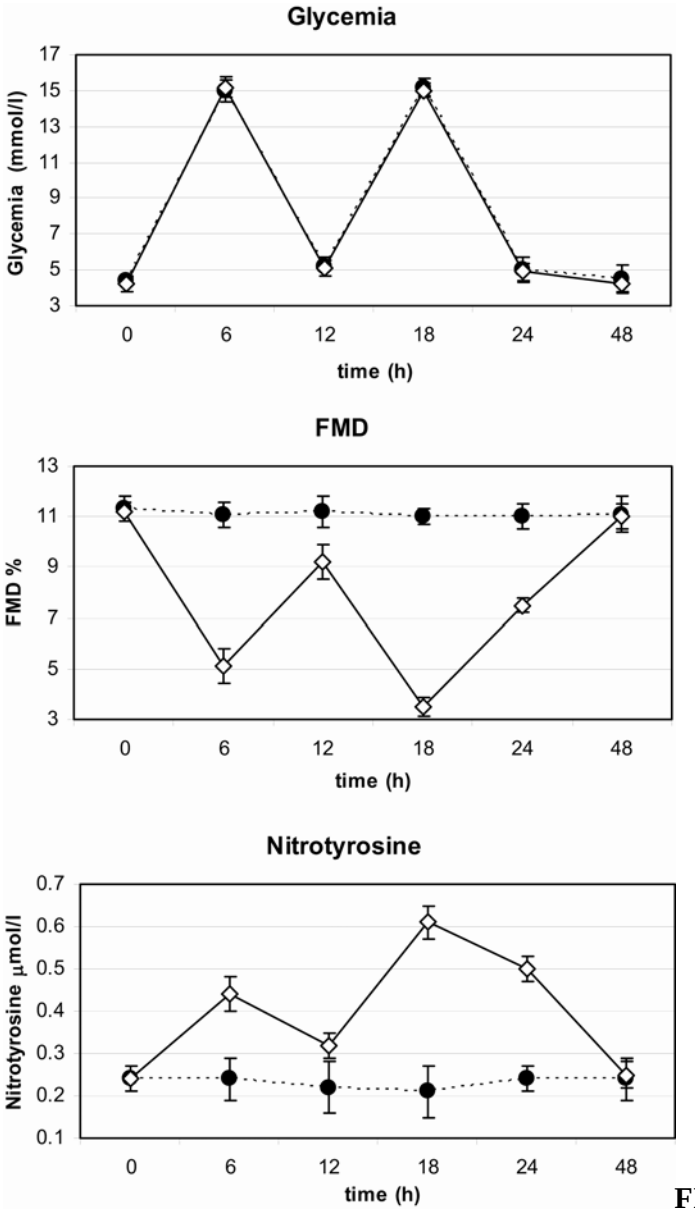
Glycemia
Glycemia (mmol/l)
17
15
13
11
9
7
5
3
0
6
12
18
24
48
time (h)
FMD
FMD %
13
11
9
7
5
3
0
6
12
18
24
48
time (h)
Nitrotyrosine
Nitrotyrosine µmol/l
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
6
12
18
24
48
time (h)

FIGURE 3

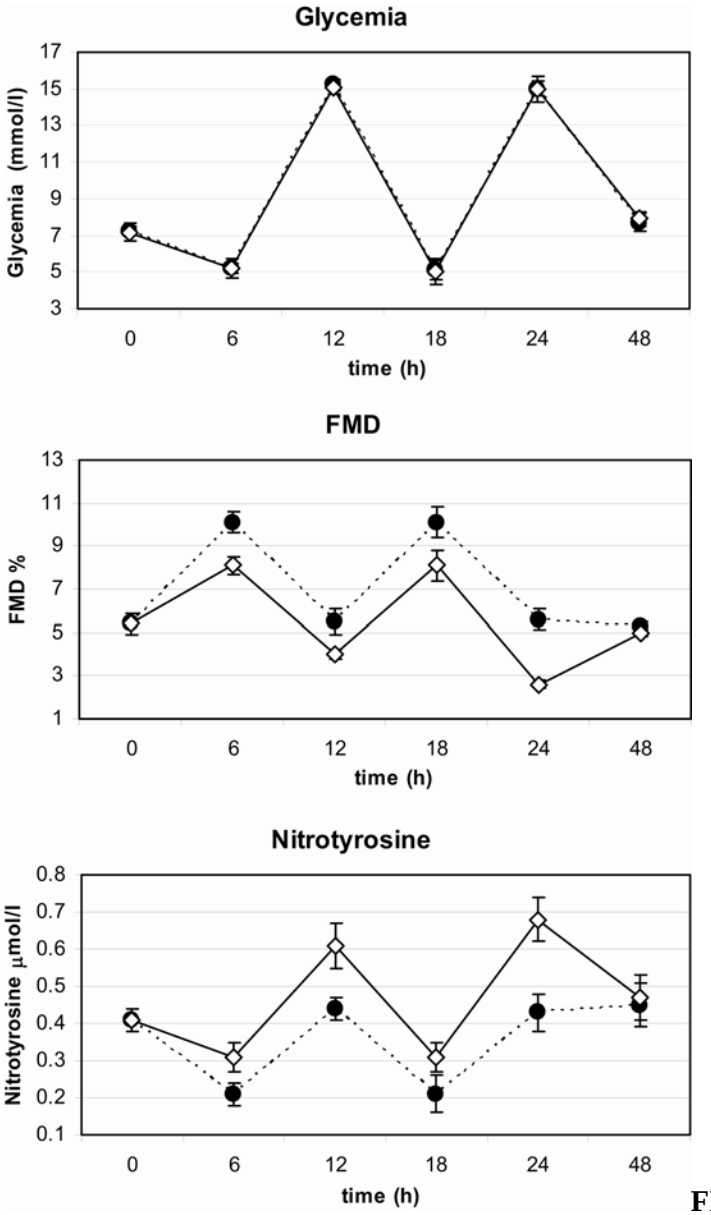
Glycemia
Glycemia (mmol/l)
17
15
13
11
9
7
5
3
0
6
12
18
24
48
time (h)
FMD
FMD %
13
11
9
7
5
3
1
0
6
12
18
24
48
time (h)
Nitrotyrosine
Nitrotyrosine µmol/l
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
6
12
18
24
48
time (h)

FIGURE 4