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# Reduction of glucose and insulin concentrations during *in vitro* incubation of human whole blood at different temperatures

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## Abstract

**Background**: Incubation of human whole blood at body temperature has been used in numerous studies without addition of glucose and insulin. The purpose of the study was to examine glucose concentration changes during *in vitro* incubation of human whole blood at different temperatures, and whether it was affected by addition of insulin and bacterial endotoxin. We also wanted to quantify changes in endogenous insulin concentrations during incubation for six hours at 37 °C.

**Methods**: Young, healthy and fasting males donated whole blood. Glucose concentrations were compared at baseline and after six hours incubation at 37 °C, 22 °C or 0 °C, in aliquots with and without addition of insulin and bacterial endotoxin. Glucose data are presented as mean (± 1 standard deviation). Endogenous insulin concentrations in aliquots without addition were measured at baseline and after six hours incubation at 37 °C, data are shown as median (interquartile range).

**Results**: Glucose concentration at baseline was 5.3 (± 0.6) mmol/L. After incubation for six hours at different temperatures, the glucose level at 37 °C was 1.0 (± 0.5) mmol/L ($p<0.01$), at 22 °C 3.0 (± 0.6) mmol/L ($p<0.01$), and at 0 °C 5.4 (± 0.7) mmol/L ($p=0.95$). The decline in glucose concentration seemed to be independent of addition of insulin and bacterial endotoxin. Endogenous insulin levels decreased from baseline 48 (36-94) pmol/L to 23 (18-27) pmol/L ($p=0.03$) during six hours incubation at 37 °C.

**Conclusions**: Glucose concentration was markedly reduced during *in vitro* incubation of whole blood from healthy volunteers for six hours at 37 °C and 22 °C, but was maintained at 0 °C. Endogenous insulin level after six hours incubation of whole blood at 37 °C was more than halved compared to baseline. During *in vitro* studies of glucose and/or insulin effects lasting for hours, measures must be undertaken to maintain stable glucose and/or insulin concentrations.

**Keywords**: Glucose, carbohydrate, insulin, hormone, human, *in vitro*, homeostasis

## Background

Changes in blood glucose levels *in vivo* may reflect underlying disturbances in the metabolic homeostasis. Hyperglycaemia and/or insulin resistance has been shown to adversely affect clinical outcomes in both diabetic and non-diabetic patients [1]. This has been confirmed for several patient categories, including pregnant women [2], patients with diabetes mellitus [3], acute myocardial infarction [4] and acute stroke [5]. Elevated glucose concentration has been associated with unfavourable outcome in both patients undergoing coronary artery bypass grafting [6] and in intensive care unit (ICU) patients [7]. Stress-induced hyperglycaemia is also a clinical relevant problem; as it has been shown to occur in a significant number of patients intraoperatively, postoperatively and during ICU stay [8].

The effects of hyperglycaemia and/or insulin resistance have been extensively studied during the last decades, utilizing both *in vivo* [9,10] and in *vitro* [11,12] models. The two mentioned methodologies are principally different as several organs (liver [13], pancreas [14], muscles [15], endothelium [16] and adipose tissue [17]) as well as regulatory mechanisms (hypothalamic-pituitary-adrenal axis [18]) influences the concentration of glucose and/or insulin in the human body, but not in isolated blood samples. Glucose and/or insulin concentration changes in aliquots during incubation might therefore be different from glucose and/or insulin homeostasis *in vivo*.

There are publications showing decreased glucose concentration during storage of blood due to glucolysis that can be inhibited by addition of antiglycolytic agents [19,20,21]. The decreases in glucose concentration seem to be dependent on both time and temperature, and result in

**Table 1. Study design: Aliquots of whole blood with different additions and measurements.**

| Aliquot incubation procedure | Number (n=) | Insulin added (nmol/L) | *E. Coli* LPS added (μg/mL) | Glucose measured | Insulin measured | Lactate measured |
|---|---|---|---|---|---|---|
| 37 °C without additions | 6 | No | No | No | 0 and 6 hours | No |
| 37 °C with additions | 6 | 30 | 1 | Every hour | No | No |
| 37 °C without additions | 8 | No | No | Every hour | No | 6 hours |
| 22 °C without additions | 8 | No | No | Every hour | No | 6 hours |
| 0 °C without additions | 8 | No | No | Every hour | No | 6 hours |

an increased lactate concentration [22]. In contrast, insulin concentration was in a previous study stable for six hours in room temperature, and 72 hours at 4°C [23]. The stability of endogenous glucose and insulin concentrations in human whole blood at different temperatures is not sufficiently quantified in previous studies; neither is the possible effect of insulin and endotoxin addition. The purpose of the study was to examine glucose concentration changes during *in vitro* incubation of human whole blood at body temperature, room temperature and placed on ice, and whether it was affected by addition of insulin and bacterial endotoxin. We also wanted to quantify changes in endogenous insulin concentrations during incubation for six hours at 37 °C.

## Methods

### Study population

Healthy males aged from 27 to 44 years donated blood after an overnight fast. The Regional Committee for Medical Research Ethics approved the study, and all donors gave oral and written informed consent before participation.

### Study design

Heparinised whole blood from each donor was divided in aliquots of 2 mL, whereof some were added insulin and *E. Coli* LPS. Aliquots were then incubated for 6 hours in an incubator (37 °C), at room temperature (22 °C) or placed on ice (0 °C). In selected aliquots glucose concentrations was measured every hour, insulin levels at baseline and 6 hours, and lactate levels after 6 hours (Table 1).

### Sampling and laboratory procedures

Venous blood from each donor was collected in tubes containing lithium-heparin 17 IU/mL blood (Vacutainer, Becton Dickinson, Plymouth, UK) and immediately placed on ice. The blood from each donor was pooled and subsequently aliquoted into polystyrene tubes with ventilation cap (Falcon, Becton Dickinson Labware, NJ, USA), each containing 2 mL blood. To selected aliquots insulin (final concentration 30 nmol/L, Actrapid, Novo Nordisk, Bagsvaerd, Denmark) and bacterial endotoxin (final concentration 1 μg/mL, *E.coli* LPS, serotype 026:B6, Difco Laboratories, Detroit, MI, USA) were added. The aliquots were incubated for 6 hours either in an incubator at 37 °C (in an atmosphere of humidified 5 % $CO_2$ and 95 % air) at room temperature (measured in blood as 22 °C) or placed on ice (measured in blood as 0 °C). Every hour the target temperature was confirmed in selected aliquots, and all tubes were agitated to obtain a homogenous solution.

### Measurements of glucose, lactate and insulin concentrations

Glucose concentrations (mmol/L) were measured every hour during incubation at 37 °C, 22 °C and 0 °C, at body temperature both in aliquots with and without addition of insulin and *E.Coli* LPS (Table 1). Glucose levels were measured utilizing Accu-Chek Sensor meter with Accu-Chek Inform test strips (Hofmann- La Roche Ltd, Basel, Switzerland).

Lactate concentrations (mmol/L) were measured in aliquots without additions after 6 hours incubation at 37 °C, 22 °C and 0 °C, respectively (Table 1). Lactate levels were measured using Arkray Lactate Pro test meter with Lactate Pro test strips (Arkray Factory Inc, Shiga, Japan). The same person did all glucose and lactate measurements on each experimental day.

Endogenous insulin concentrations (pmol/L) were measured in aliquots without additions at baseline and after 6-hour incubation at 37 °C (Table 1). Insulin measurements were performed in plasma after centrifugation at 2000 g for 12 minutes at 4 °C and storage at – 70 °C. Insulin was measured by a radioimmunoassay kit (Linco Research Inc, St. Charles, MO, USA), intra-assay coefficient of variation was<5 %.

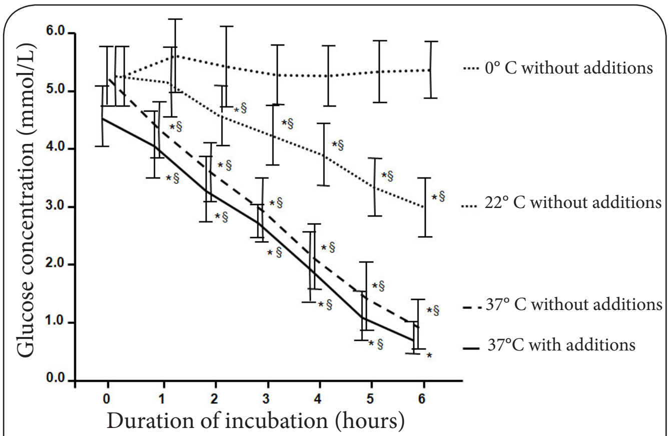


**Figure 1.** Glucose concentrations during incubation of human whole blood for 6 hours.
Different aliquots were placed at 37°C with additions (n=6), 37°C without additions (n=8), 22°C without additions (n=8) and 0°C without additions (n=8). Additions were insulin 30 nmol/L and E. Coli LPS 1 μg/mL. Glucose concentrations every hour during incubation are presented as line chart of means with error bars of ± 1SD. Paired T-test was used to compare data from each measuring hour with baseline (*=p<0.05) and from one hour to the next hour (§=p<0.05).

**Table 2. Lactate concentrations (mmol/L) after incubation of human whole blood for 6 hours. Paired T-test was used to compare aliquots incubated at 37 °C versus 22 °C (*=p<0.05) and aliquots incubated at 22 °C versus 0 °C (§=p<0.05).**

| Person number | 37 °C | 22 °C | 0 °C |
|---|---|---|---|
| 1 | 7.1 | 3.8 | 1.0 |
| 2 | 8.8 | 4.4 | 1.1 |
| 3 | 8.2 | 4.2 | 1.0 |
| 4 | 7.6 | 3.7 | 0.8 |
| 5 | 8.0 | 3.8 | 1.4 |
| 6 | 7.8 | 3.6 | 0.9 |
| 7 | 8.0 | 4.3 | 1.3 |
| 8 | 9.9 | 4.4 | 1.2 |
| Mean (±SD) | 8.2 (±0.8) * | 4.0 (±0.3) * § | 1.1 (±0.2) § |

## Statistical analysis

Glucose and lactate data showed a normal distribution and are presented as mean (± 1 standard deviation (SD)). One-way ANOVA was used to analyze significant effects of time and/or temperature on glucose concentrations. Paired T-test was used to test the significance of a decline in glucose levels during incubation. Independent sample T-test was utilized to compare glucose concentrations at different temperatures and with different additions. Insulin data showed a skewed distribution and are presented as median (interquartile range (IQR)). Wilcoxon signed rank test was utilized to compare insulin concentrations at baseline and 6 hours. Statistical analyses was performed utilizing Statistical Package for Social Sciences (SPSS), version 15.0 (SPSS Inc.,Chicago, IL, USA). Significance was accepted at p<0.05.

## Results

### Glucose concentrations

At 37 °C the blood glucose concentration in aliquots without additions declined from 5.3 (± 0.6) to 1.0 (± 0.5) mmol/L after 6 hours (p<0.01); the mean decline was 0.7 (± 0.2) mmol/L/hour, which was significant for each time interval. The decline in aliquots with additions was 0.6 (± 0.3) mmol/L/hour, which was significant for each time interval except for from 5 to 6 hours. Independent samples T-test revealed that the decline in glucose concentrations was not significantly affected by additions of LPS and insulin (**Figure 1**).

At 22 °C the blood glucose concentration declined from 5.3 (± 0.6) to 3.0 (± 0.6) mmol/L after 6 hours (p<0.01); mean decline was 0.4 (± 0.2) mmol/L/hour, which was significant for all time intervals except between 0 and 1 hour (**Figure 1**).

At 0 °C there was no detectable decline in glucose concentration during the incubation period as glucose level was 5.4 (± 0.7) mmol/L after 6 hours (p=0.95) (**Figure 1**).

### Lactate concentrations

At 37 °C, lactate concentration was 8.2 (± 0.8) mmol/L after 6 hours incubation, at 22 °C 4.0 (± 0.3) mmol/L and at 0 °C 1.1 (± 0.2) mmol/L. Paired T-test revealed a significant difference in lactate level between 37 °C and 22 °C (p<0.01), and between 22 °C and 0 °C (p<0.01) (**Table 2**).

### Insulin concentrations

Wilcoxon signed rank test revealed that endogenous insulin concentration after incubation at 37 °C for 6 hours was significantly reduced from baseline 48 (36-94) to 23 (18-27) pmol/L (p=0.03) (**Figure 2**).

## Discussion

The main finding in this study was that glucose concentration was reduced to almost zero during 6 hours *in vitro* incubation of whole blood at body temperature. The decline in glucose concentration was independent of addition of insulin and bacterial endotoxin. At 22 °C glucose level was reduced by approximately 50 % of that at 37 °C after 6 hours, while at 0 °C glucose concentration was maintained. There was also 52 % lowered endogenous insulin concentration at 6 hours and 37 °C compared to baseline.

Glucolysis is known to cause falling glucose concentrations in blood samples after collection, and antiglycolytic agents can be added in order to maintain glucose levels [**19**,**20**,**21**]. Such antiglycolytic agents can interfere with cellular functions, and are therefore not used during studies of activated blood cells. Measurements of glucose concentrations during incubation of blood with different glucose additions have usually not been performed in previous studies [**11**,**12**], only the measured and/or calculated glucose level at baseline

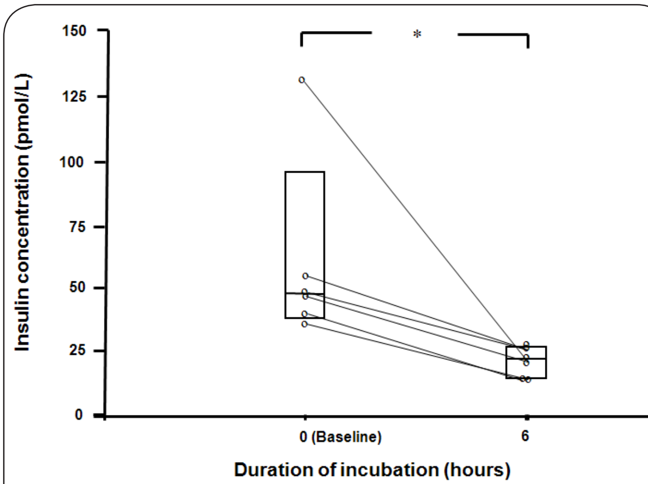


**Figure 2.** Insulin concentration during incubation of human whole blood at 37°C for 6 hours.
Insulin level before and after incubation are presented as box plots with medians in 25th and 75th percentile boxes in addition to individual values. Wilcoxon signed rank test was used to compare data from 6 hours with baseline (n=6) (*=p<0.05).

has been reported [11,12]. The present study revealed an almost linear, temperature-dependent decline in glucose concentration during incubation at body temperature, down to severely hypoglycaemic levels after 6 hours. As erythrocytes obtain their metabolic energy by glycolysis, and the same effect was observed in aliquots with and without insulin additions, we propose that the decline was independent of any effects of insulin [24]. This hypothesis is supported by the measured lactate concentration after 6 hours incubation, as lactate is the metabolic end product of glycolysis with 2 molecules of lactate generated for each molecule of glucose.

The addition of insulin has also been done in numerous *in vitro* experiments without measurements of insulin concentration during incubation. Insulin level at baseline is often reported and assumed to remain constant during incubation. In contrast, we found in the present study that the median endogenous insulin concentration was reduced by more than 50 % during 6 hours incubation at 37 °C. This was unexpected, as insulin is mostly degraded in liver and kidney, but to some extent also in blood cells and extracellularly [25]. Insulin concentrations in normal whole blood has in previous studies been stable at room temperature for 5 hours [26], at 37 ºC insulin have been reported to be bound to human erythrocytes followed by subsequent internalization and degradation [27,28]. Thus, insulin is degraded by viable erythrocytes at 37 ºC while the erythrocytes are unable to degrade insulin at room temperature, presumably due to reduced metabolic activity at this temperature.

This study contributes new knowledge regarding glucose and insulin degradation in whole blood at different temperatures. Even if our samples were examined *ex vivo,* the same rate of degradation by blood cells can be expected to occur *in vivo*. Under normal circumstances, surrounding tissues replenish blood glucose and insulin continuously. When the glucose and glycogen reserves are exhausted, also the blood cells participate in creation of a hypoglycaemic state with degradation rates calculated from our results to be close to 20 g glucose/24 hours, which is in agreement with data from others [29,30]. Based on our results, we suggest that former publications regarding effects of glucose and/or insulin during *in vitro* incubation should be interpreted with caution, as the reported glucose and/or insulin concentrations may not be representative for the entire incubation period. To avoid hypoglycaemia we also suggest that glucose or other energy supply for cells should be added in future *in vitro* studies lasting for hours. Further, when glucose and/or insulin effects are studied during incubation, measures should be undertaken to maintain stable glucose and/or insulin concentrations.

A limitation was the use of only one glucose measurement method as the accuracy of different glucose measurement techniques has lately been elucidated [31]. The present study does not deal with the mechanisms behind decline in glucose and insulin concentrations, the respective contributions of erythrocyte glycolysis and leukocyte metabolism has to be addressed in future studies. The main goal of this investigation was to examine glucose dynamics in relation to a commonly used method for examining various aspects of leukocyte function after activation *ex vivo*, *i.e.,* by incubating whole blood at body temperature (37 °C) for several hours. As addition of insulin and bacterial endotoxin did not affect the glucose metabolism at 37 °C, it was unlikely to do so at lower temperatures. A comparison of insulin degradation at different temperatures could have been interesting, but the dynamics of insulin in blood *per se* was not focused upon in this investigation. Furthermore, the effect of exogenous glucose addition on glucose degradation during incubation should be addressed in future studies.

## Conclusions

There is a considerable degradation of glucose in whole blood at body temperature. During *in vitro* incubation of whole blood from healthy volunteers, glucose concentration was markedly reduced, after 6 hours incubation down to almost zero. Reduction of glucose concentration seemed to be independent of addition of insulin and bacterial endotoxin. There was also significantly lowered endogenous insulin level after incubation of whole blood for 6 hours at 37 ºC.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

HO and TL contributed to the conception and design of the study. SB recruited persons, performed the glucose and lactate analyses, and drafted the manuscript. TA was responsible for blood sample collection and handling, including the incubation procedure. PAT was responsible for the insulin concentration measurements. SB, HO and TL analyzed and interpreted data, and all authors read and approved the final manuscript.

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