Thesis

Evaluation of Arterial Blood Glucose Measurements in a Porcine Model

submitted by

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Graz, July 18th, 2016

Statutory Declaration

I declare on my honor that I have written this thesis independently and without assistance, I have not used other than the specified sources and parts taken from other sources, verbatim or in substance have been identified as such.

Graz, July 18th, 2016 *Alexandru-Cristian Tuca eh.*

Note of thanks

I would like to thank my advisor, Priv.-Doz. Dr. Stefan Korsatko, MBA for guiding and supporting me over the years. You have set an example of excellence as a researcher, mentor, instructor, and role model.

I would like to thank my amazing family for the love, support, and constant encouragement I have gotten over the years. In particular, I would like to thank my parents and my brother.

Finally, I would especially like to thank my girlfriend Vera. You are the salt of the earth, and I undoubtedly could not have done this without you. You are the moon of my life.

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Glossary and Abbreviations

ICU	Intensive care unit
BGA	Blood gas analyzer
POC	Point of care
CLINICIP	Closed loop insulin infusion for critically ill patients
GIR	Glucose infusion rate
ECG	Electrocardiography
CRF	Case report form
GCP	Good clinical practice
AE	Adverse event
PTT	Partial thromboplastin time
SD	Standard deviation
I.U.	International units
CV	Coefficient of variation

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Zusammenfassung

Einführung

Gute Blutzuckerkontrolle ist essentiell für PatientInnen mit Diabetes. Wissenschaftliche Erkenntnissen zeigen, dass auch PatientInnen im Krankenhaus unter stressinduzierten Hyperglykämie leiden können. Im Laufe der letzten Jahrzehnte wurden daher erhebliche Anstrengungen zur Entwicklung von Glukose-Biosensoren unternommen, welche sicher und effizient unter unterschiedlichen Bedingungen verwendet werden können. Aufgrund der hohen Sicherheits- und Qualitätsanforderungen im Gesundheitswesen ist es notwendig, diese Glukose-Biosensoren in einer effektiven und sicheren vorklinischen Umgebung zu evaluieren. Diese vorklinische Umgebung sollte die Genauigkeit der Sensoren in verschiedenen Glukosebereichen (hypo-, normo- und hyperglykämisch) in vivo testen können. Ein ideales Modell für diesen Zweck könnte jenes im narkotisierten Schwein darstellen. Das Ziel dieser Diplomarbeit war es, die Machbarkeit eines präklinischen Schweinemodells für die Untersuchung von Blutzucker -Biosensoren zu evaluieren.

Material und Methoden

In der vorliegenden Diplomarbeit wurden 10 gesunde Schweine drei unterschiedlichen Glucose und Sauerstoff Clamp-Protokollen (A, B und C) unterzogen. Diese wurden in einer 10 stündigen Vollnarkose durchgeführt. Zwei arterielle Katheter wurden in die inguinalen Arterien gesetzt und zur Blutentnahme verwendet, um Referenz- und Biosensormessungen durchzuführen. Die individuellen Clamp-Profile wurden anschließend unter Verwendung des Variationskoeffizienten (VK) ausgewertet.

Ergebnisse

In Clamp-Protokoll A betrug der mittlere VK im Glukosezielbereich von 100 mg / dl, 8,01% ± 3,78%, der mittlere VK im Glukosezielbereich von 40 mg / dl, 19,65% ± 11,09% und der mittlere VK im Glukosezielbereich von 200 mg / dl, 8,98% ± 6: 45%. In Clamp-Protokoll B, betrug der mittlere KV im Glukosezielbereich von 100 mg / dl, 15: 06% ± 19,75%, der mittlere KV im Glukosezielbereich von 40 mg / dl 8,68% ± 2 58% und der mittlere KV im Glukosezielbereich von 200 mg / dl,

13.23% ± 9,63%. Der mittlere VK für Clamp- Protokoll C, im Glukosezielbereich von 100 mg/dl, betrug die 6,99%.

Schlussfolgerungen

Die Experimente zeigen, dass das vorliegende Modell im narkotisierten Schwein noch einige Verbesserungen bezüglich der Clamp-Qualität benötigt.

Bei Berücksichtigung der notwendigen Verbesserungen ist das Modell jedoch in der Lage ein sicheres und effektives präklinisches Umfeld zu schaffen, in der es möglich ist Glukose-Biosensoren in variierenden Glukose- und Sauerstoffkonzentrationen zu untersuchen.

Abstract

Introduction

Glucose control is of importance for diabetic patients at home, but according to recent findings also for patients in the hospital or for critically ill patients suffering from stress induced hyperglycaemia. Over the last decades, substantial effort has been made towards the development of glucose biosensors that can be efficiently and safely used in different settings. Due to increased safety and quality demands in health care it is necessary to evaluate these glucose biosensors in a safe and effective preclinical setting. This setting should be able to test the sensors' accuracy in different glucose ranges (hypo-, normo- and hyperglycaemic) in vivo. An ideal model for this purpose could be the anesthetized porcine model. The aim of this thesis was to evaluate the feasibility of a preclinical porcine model for the investigation of blood glucose biosensors.

Material and Methods

In the present thesis 10 healthy pigs underwent three different glucose and oxygen clamp protocols (A, B and C) under general anaesthesia for 10 hours. Two arterial catheters placed into the inguinal arteries were used for blood sampling to perform reference measurements and biosensor measurements. The clamp profiles were evaluated using the coefficient of variation (CV), a parameter which describes the quality of the glucose clamps.

Results

In clamp protocol A, the mean CV of glucose target 100 mg/dl was 8.01%±3.78%, the mean CV of glucose target 40 mg/dl 19.65%±11.09% and the mean CV of glucose target 200 mg/dl was 8.98%±6.45%. In clamp protocol B, the mean CV of glucose target 100 mg/dl was 15.06%±19.75%, the mean CV of glucose target 40 mg/dl 8.68%±2.58% and the mean CV of glucose target 200 mg/dl was 13.23% ±9.63%. Mean CV for clamp protocol C during the 10 hour normoglycemic (100mg/dl) period was 6.99%.

Conclusions

The experiments indicate that the present anesthetized porcine model still needs improvements concerning the clamp quality. Nevertheless, taking these into account the present anesthetized porcine model is capable to provide an safe and effective preclinical environment for evaluation of glucose biosensors in varying glucose and oxygen ranges.

1 Introduction

1.1 Scientific Background and Rationale

1.1.1 General

Until 2001, research concerning blood glucose control and insulin therapy was primarily performed in connection with diabetes type 1 and type 2. This changed drastically in 2001, after a publication by Greet van den Berghe et al, indicating that blood glucose normalization in critically ill patients at the intensive care unit (ICU) could improve clinical parameters like mortality and morbidity (1). The Greet van den Berghe paper led to intense worldwide scientific discussion and research inducing several important manuscripts supporting or objecting their results (2–4). Shortly after the first publication, Greet van den Berghe et al also showed that mortality of high-risk cardiac surgery patients with intensive insulin control during the ICU was reduced from 9.9% down to 3,4% (5).

Apart from the discussion about targets and intensity of blood glucose control at the hospital wards, at least tight blood glucose monitoring has become a standard hospital procedure. As we know from daily routine, at the moment blood glucose monitoring intervals vary from 2-3 times daily at general wards up to 10-15 measurements or more within 24 hours at intensive care units. At the ICU, the measurements are often performed in a manual way using blood gas analyzers (BGA) or modern ICU point of care (POC) glucose meters, a rather time consuming approach. Another problem is that blood glucose values have to be evaluated by a physician, again binding significant resources. The physicians' decision commonly happens intuitively and mostly based on experience.

In order to decrease workload at the ICU and to establish methods, which assess blood glucose values objectively and provide glucose control, scientific effort has been made towards the development of closed loop glucose control systems including the research of biosensors and algorithms.

1.1.2 Closed Loop System

“The closed loop insulin infusion for critically ill patients (CLINICIP) system is a low-risk monitoring and control system for metabolic control in critically ill patients. The core of the system is a computer algorithm implemented into an ICU infusion system, which calculates insulin dosage from metabolic parameters to provide decision support for tight glycaemic control. A glucose sensor and a body interface have been integrated to allow for closed-loop insulin infusion“ (6).

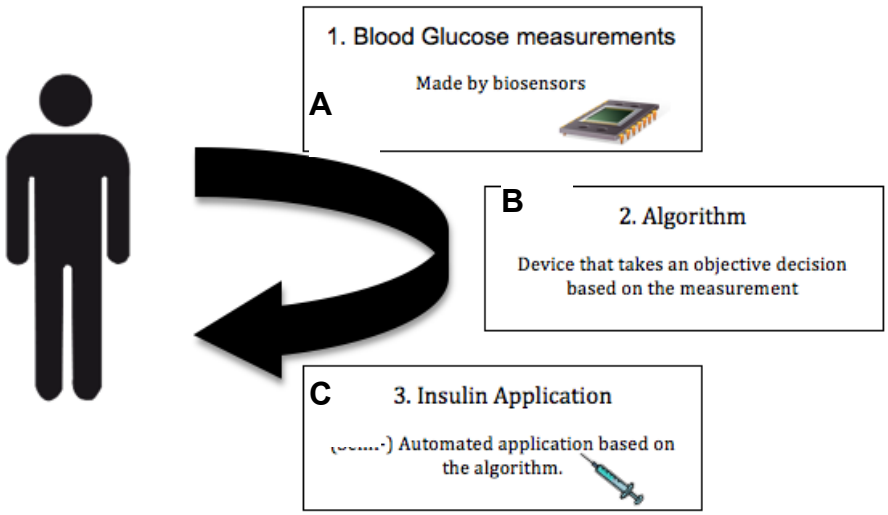

Figure 1 Schematic of the closed loop set-up.

In Figure 1 a schematic overview is shown which describes the set-up of the closed loop system: A biosensor measures continuously (placed e.g. intravenously, intra-arterially or subcutaneously) in a predefined interval the blood glucose to be able to feed an algorithm with the blood glucose values (see Figure 1 A). Subsequently, an algorithm (see Figure 1 B) analyses the values and calculates an objective recommendation for the further insulin administration. The Insulin administration can be automated, this means the system decides by itself if it accepts the recommendation; or semi-automated, which means a physician has to confirm the insulin therapy recommendation (see Figure 1 C).

1.1.3 Rationale

Apart from the computer algorithm, the glucose sensor itself has a crucial role because all calculations concerning recommendation for the insulin administration are based on its accuracy. Consequently, a sensor which does not work accurate and reliable could lead to life threatening false recommendations. These include low blood glucose values as well as high ones where the organism can react with tachycardia, arterial hypertension, hyperventilation, unconsciousness, polyuria and many other symptoms that would strain the organism of critically ill patients.

Before sensors can be used at hospital wards and especially at ICUs, they have to be tested in clinical and preclinical trials with regard to accuracy, reliability and functionality; as well at high blood glucose ranges as at low blood glucose ranges. These blood glucose excursions are necessary to evaluate the biosensors' ability to follow rapidly changing values and its accuracy in hypo-, normo- and hyperglycemic ranges.
Depending on the biosensors principle for glucose detection also other parameters have to be investigated in clinical and preclinical trials. For example, for those which work in dependence on blood oxygenation, like many optical sensors, need also to be tested in different ranges of blood oxygen to determine the influence of low or high blood oxygenation values on their glucose measurement accuracy.
Blood oxygenation is also a critical and highly variable parameter in critically ill patients, thus any influence on blood glucose sensing could lead to an erogenous result and further to life threatening decisions during blood glucose regulation.

The present thesis is the result of a study in which prototypes of glucose biosensors were tested in a preclinical environment. The aim of this thesis is to describe the feasibility of a preclinical porcine model for the investigation of blood glucose biosensors. Furthermore, it describes the various requirements but also limitations of the porcine model. This thesis does not describe the biosensors and their performance in the study.

1.2 Objectives

1.2.1 Primary objective

The primary objective of the present thesis is to evaluate the feasibility of a preclinical porcine model for the investigation of blood glucose biosensors.

1.2.2 Study model specific objectives:

- Investigation of the reproducibility of the experiments.
- Investigation of blood glucose and blood oxygen profiles.

2 Material and Methods

2.1 Animals

The preclinical study for the present thesis was performed in 10 healthy domestic pigs (Latin: suus domesticus) delivered by a local farmer. The animal experiments were approved without any objections by the Animal Care and Use Committee at the Veterinary University of Vienna on behalf of the Austrian Ministry of Science and Research. We chose domestic pigs in order to be able to take a sufficient amount of blood; therefore, smaller animals were not suitable for this study. Following the animal delivery, the pigs were kept 4-7 days at the animal facility of the Institute for Biomedical Research at the Medicals University of Graz, Hahnhof 48, 8036 Graz, in order to assure their adaptation to the new environment.

2.2 Ethical Considerations

In these animal experiments, a newly developed glucose sensor was tested with regard to feasibility, stability and also to accuracy in comparison to reference measurements. The acquired data should increase the potential for further development and improvement of the sensor. Thanks to previous intensive in vitro testing the number of animal experiments was minimized. The structured comprehensive experimental plan was able to bring maximal knowledge on accuracy and efficacy of the sensor that can be efficiently used for further development and thus reduce the number of necessary human experiments. Moreover, these animal experiments reduced the hazards for future human studies, because some of the potential side effects could be recognized already within this study.

Within the experiments the animals did not suffer from because all the interventions were performed under general anesthesia.

2.2.1 Justification of the animal model experiment

The glucose sensor had to be tested in whole blood under conditions adequate to final intended use. The in vitro experiments could only be performed in heparinized blood samples that do not entirely mimic the biological conditions of intended use. Moreover, human experiments are exclusively possible after completion of toxicological tests which makes a development of such a sensor impossible. The complete clinically relevant ranges of glucose concentrations could only be tested in an animal model.

2.2.2 Justification of animal species used for experiment

In glucose clamp experiments there are frequent blood samplings necessary. This represents a total blood volume of ca. 240 ml (2 arterial catheters, each with 120 samples of 1 ml) that can be safely withdrawn only in a larger animal model. In order to ensure the testing of the glucose sensor according to its intended use, there is also a need to provide the catheter system corresponding to its use in humans. The pig was chosen as an animal model for this experiment with regard to both of these reasons.

2.2.3 Animal Preparation and Anesthesia

Before the start of the experiments, a qualified veterinarian administered the pre-medication to the pigs for making them calm and sleepy in order to be able to insert an intravenous catheter into an ear vein for anesthesia medication. Narcosis was introduced intravenously or via inhalation depending on the respective veterinarian doctor. Before starting anesthesia, the animals were pre-oxygenated with 100% O_2 for 3 to 5 minutes. After anesthesia start and intubation two arterial catheters for arterial blood sampling were inserted into the inguinal arteries. Arterial blood lines were necessary for reference measurements (see Figure 2). Respectively one on the left side for reference measurements and one on the right side for biosensor measurement. Next, a central venous catheter was inserted into the surgically accessed jugular vein for further medication infusion and for monitoring of central venous pressure (see Figure 3). Afterwards, all necessary

further equipment was fixed and checked for proper function. This included the electrodes for electrocardiography (ECG), suprapubic urine catheter, connections to the anesthesia machine, arterial blood sampling set and saline solution for draining the arterial catheters in order to avoid clogging and subsequent failure of the arterial line (see Figure 2). Finally, after all other preparations were finished the biosensors were affixed properly (further information about the affixation of the biosensors can not be given due to a confidentially agreement) and the vital signs of the animals were monitored continuously.

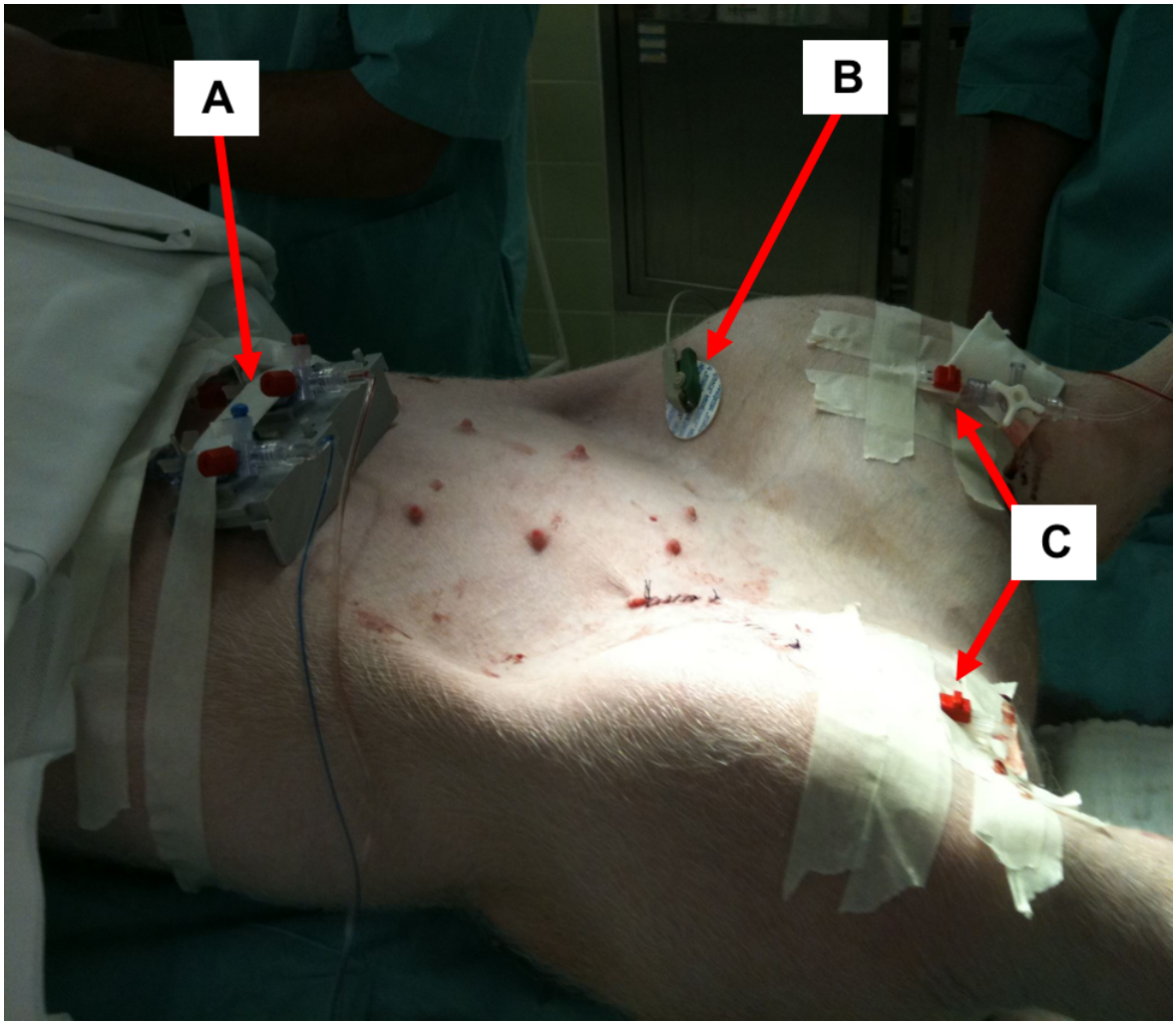

Figure 2 Arrangement of the study material: Shown are a part of the blood sampling set (A), ECG electrodes (B).and arterial blood catheters (C),

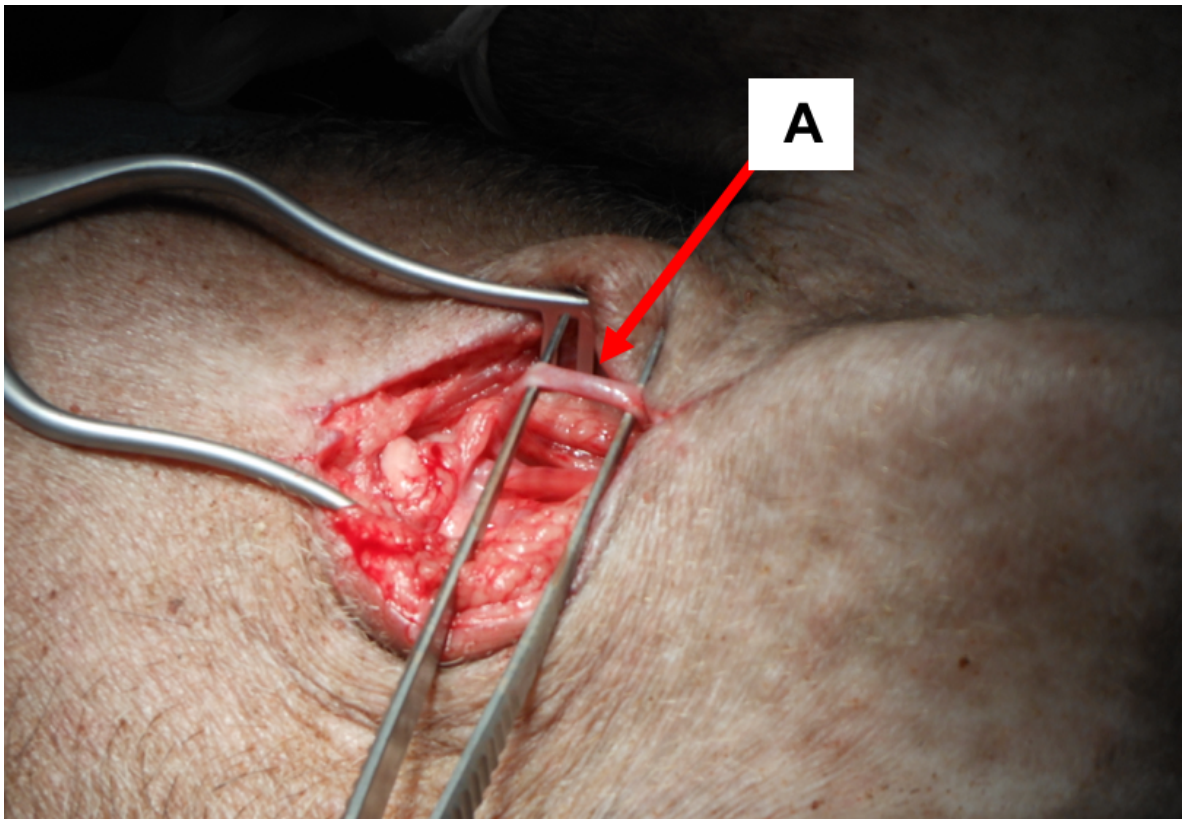

Figure 3 Central venous vein: Shown is the surgical access to the jugular vein (A) for insertion of a catheter, medication infusion and central venous pressure measurement.

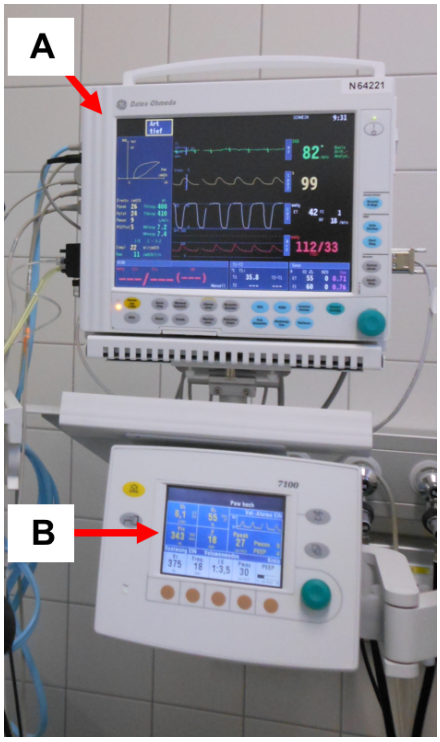

Figure 4 Anesthesia machine monitors: Shown are the monitors of the anesthesia machine by which the pigs' vital signs were monitored. Displayed are heart rate, blood pressure, blood oxygenation and lung ventilation (A). Furthermore, the anesthesia machine comprises a control unit for the anesthetic gas supply and oxygen supply(B).

2.2.4 Animal Medication

2.2.4.1 Medication Related to Anesthesia

Animal medication was performed and adjusted by an experienced anesthetist who was continuously present in the operation theatre.

2.2.4.2 Heparin and Heparin Administration

2.2.4.2.1 Heparin

Heparin occurs naturally in the human body and serves as an anticoagulant which is produced by mast cells and basophils (7). This means it prevents the formation of blood clots and their extension; it does not dissolve already build clots. Heparin can also be administered as a medication for anticoagulation.

2.2.4.2.2 Heparin Administration

“Blood vessels in swine are proportionately smaller than those in humans on a body-weight basis” (8). This means the blood vessels of pigs are more fragile than human blood vessels because the wall-thickness is smaller and so the risk of rupture is much bigger. Additionally, the coagulation time of porcine blood is twice faster than in humans (9). Thus, after the third experiment we decided to administer heparin to avoid failure of the arterial lines, to prevent coagulation and to reduce the impact of frequent blood aspirations, which were done every 7.5 minutes on the same artery. Further information concerning administered heparin doses and why heparin administration started after the third experiment are described in the results section, point 3.3.3.

2.2.4.3 Insulin and Insulin Infusion

2.2.4.3.1 Insulin

Insulin is an anabolic peptide hormone and synthesized in the beta cells of the pancreatic islets of Langerhans (10,11). It is used for lowering the blood glucose levels in patients e.g. with diabetes type I, type II or in critically ill patients with major blood glucose excursions and consequently suffering from hyperglycemia (1,12). Insulin can also be used for research matters in order to control or 'clamp' the blood glucose but also to quantify beta-cell sensitivity to glucose and tissue sensitivity to insulin (13).

2.2.4.3.2 Insulin Infusion

A variable rate of intravenous insulin infusion was used to achieve different glycemic levels, especially the hypoglycemic ones by using the ability of insulin to lower the blood glucose. More information can be found in the Clamp section, point 2.2.7.

2.2.4.4 Glucose and Glucose Infusion

2.2.4.4.1 Glucose

Glucose is a simple sugar (monosaccharide) and has the molecular formula $C_6H_{12}O_6$. In the human body it used by the cells as fuel for energy. During blood glucose clamps it used to control the blood glucose.

2.2.4.4.2 Glucose Infusion

A variable rate of intravenous glucose infusion was used to achieve and maintain different glycemic levels. More information can be found in the Clamp section, point 2.2.7.

2.2.4.5 Potassium and Potassium Supplementation

2.2.4.5.1 Potassium

Potassium is a chemical element and electrolyte and has a key role in multiple physiological processes like:

- Vascular tone (14)
- Blood pressure control (15)
- Acid – base homeostasis (16)
- Fluid and electrolyte balance (17)

2.2.4.5.2 Potassium Supplementation

Insulin stimulates cellular potassium uptake, consequently the blood potassium level decreases (18,19). Due to the high insulin administration during experiments (see clamp section, point 2.2.7.1.1) and depending on the blood potassium levels, determined by the BGA, potassium was administered to balance blood potassium levels.

2.2.5 Biosensor Measurements

A run-in period was needed for stabilization of the biosensors. After the run in period of approximately 30 minutes the measurements were started. Biosensor measurements were performed in 7.5-minute interval.

2.2.6 Sample Collection and Analysis

2.2.6.1 Sample Collection

A run-in period was also needed for stabilization of the animals' blood glucose and blood oxygen values. After the run in period of approximately 30 minutes the measurements were started. Sampling for reference and reference measurements were performed in 7.5-minute interval using a blood sampling set with a piston. Additionally, blood sampling for electrolyte (i.e. potassium levels), blood oxygen concentration was done various times in the course of the experiments.

The arterial blood sampling set comprises of various functional parts:

- Arterial catheter, which is inserted into an inguinal arterial vessel (see Figure 5 D).
- Septum, which is used to aspirate blood out of the line (see Figure 5 C).
- Piston, which is used to suction blood out of the arterial vessel and to push it back; it ensures that the blood is not diluted (see Figure 5 A)

For blood sampling the piston was pulled up and arterial blood was suctioned out of the artery into the line. Then a 2 ml syringe with an adapter was pushed into the septum by which it was possible to aspirate 1ml arterial blood from the line. Finally, the piston was pushed back down to push the blood back into the artery and the line was flushed with saline solution to avoid clogging (see Figure 5).

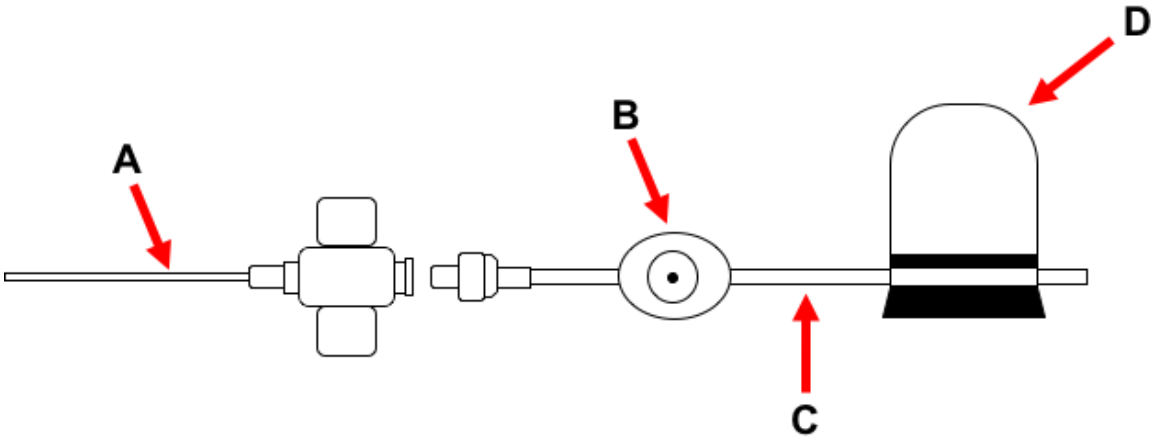

Figure 5 Arterial line: Shown are the arterial catheter (A) and the blood sampling set. The blood is aspirated with a syringe through the septum (B). After finishing use, the line is always flushed with saline solution to keep it clean and free from coagulation clots (C). By pulling or pushing the piston up or down the blood is aspirated or pulled back into the line and artery (D).

2.2.6.2 Sample Analysis

After blood sampling the samples were transferred immediately from the syringe into lithium heparin coated tubes to avoid coagulation. Next, the sample was centrifuged (see Figure 6 B) for 1 minute and the glucose concentration was determined in the supernatant using a Beckman Coulter Glucose Analyzer 2 (Beckman Instruments, Inc., Fullerton, CA, USA), a glucose-oxidase based measurement device (see Figure 6 A and Figure 7 A).

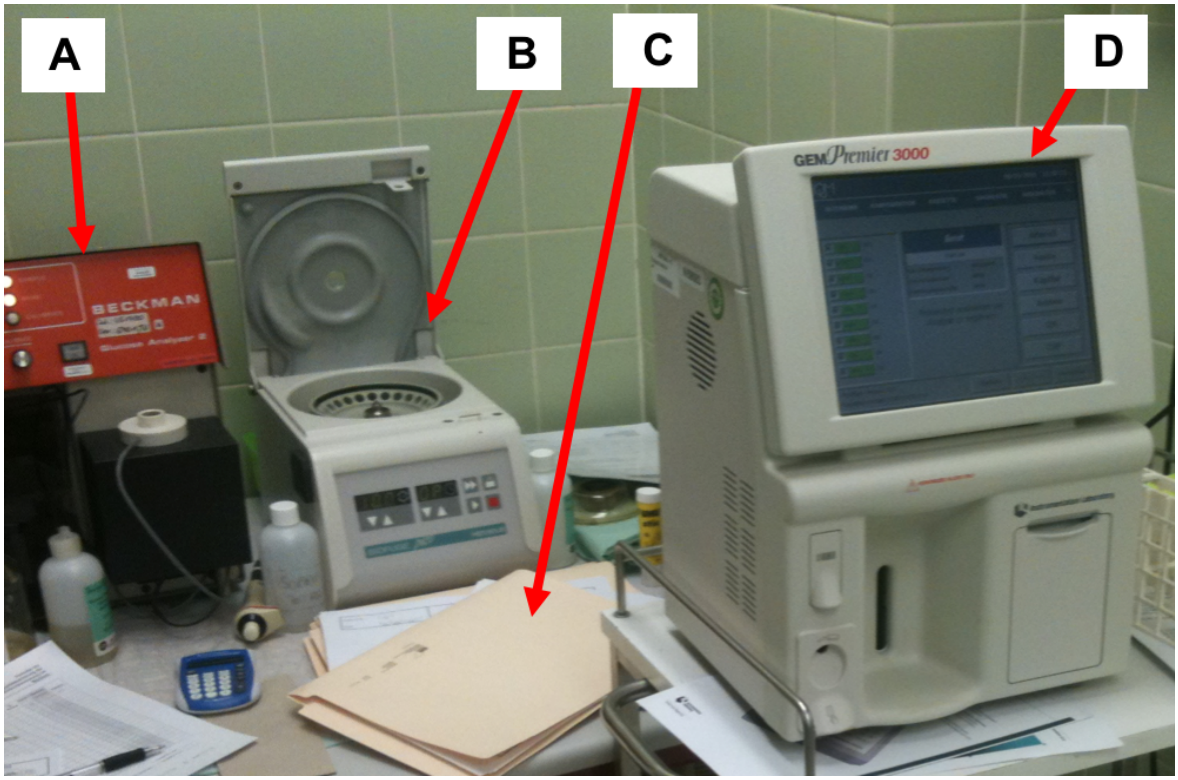

Figure 6 Centrifuge and BGA: Shown are again the glucose analyzer (A). Furthermore, the centrifuge (B) and for data recording a case report form (C) was used. The BGA (D) is pictured too.

Additionally, hourly BGA analyses with a GEM Premier 3000 (Instrumentation Laboratories, Lexington, MA, USA) were performed in the course of the experiments (see Figure 6 D). They were needed to determine the current potassium levels and the results of the analyses were used as reference for the oxygen clamp.

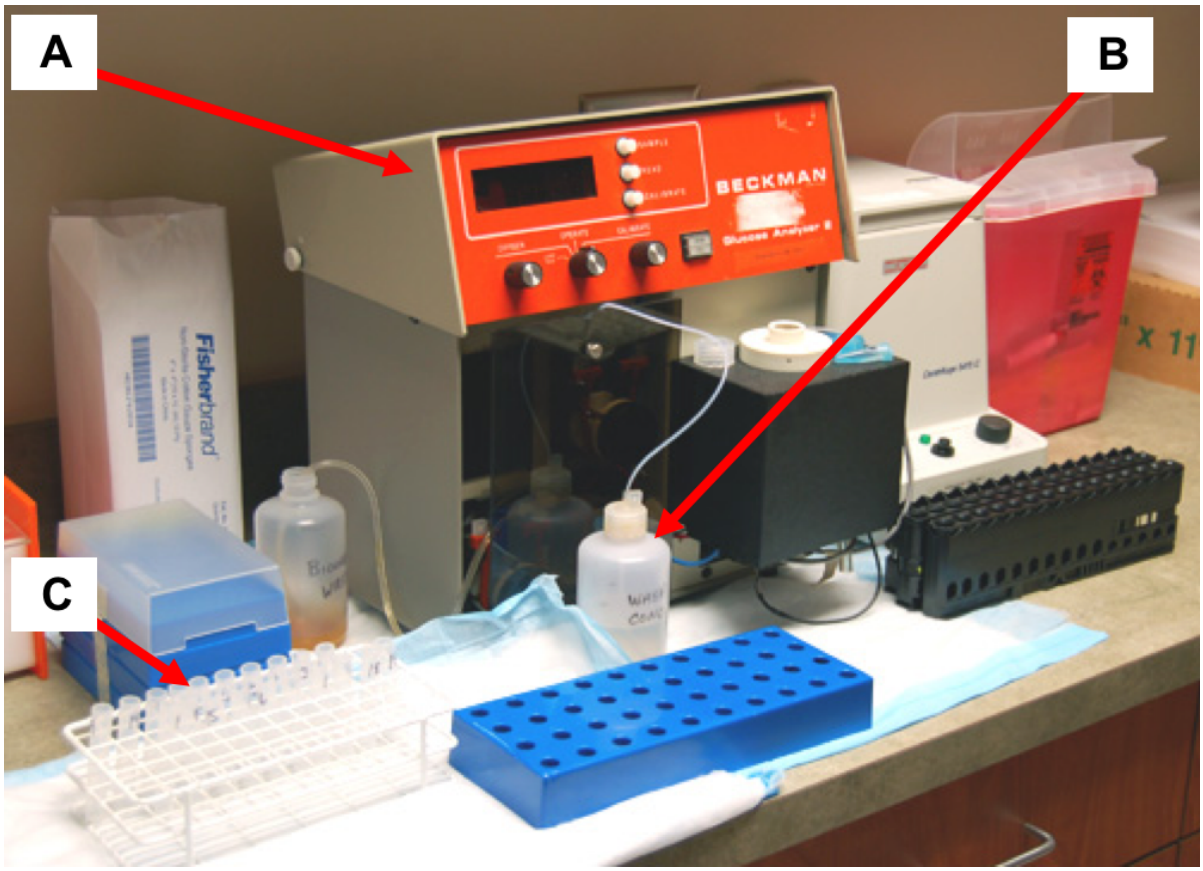

Figure 7 Glucose analyzer: Shown are the Beckman Coulter Glucose Analyzer 2 (A),its reagents for measurement (B) and a holder for the test samples (C).

2.2.7 Glucose Clamp and Oxygen Clamp

In order to produce different blood glucose and blood oxygen concentrations in clinically relevant measurement ranges, the glucose clamp technique and oxygen clamp technique was used (see e.g. Figure 8 and Figure 9)

2.2.7.1 Glucose Clamp

The glucose clamp technique is a highly reproducible method for quantification of beta-cell sensitivity to glucose and of tissue sensitivity to insulin. It allows the calculation of the metabolic clearance rate as well as the basal systemic delivery rate of insulin (13). Primarily, it is a scientific method used for drug and physiological (insulin, glucagon) investigations (20,21). However, the glucose clamp technique can be also used just to produce varying glucose concentrations in order to evaluate glucose biosensors. During the experiments following three types of glucose clamps were used with disregard to the physiological events:

- Hypoglycemic hyperinsulinemic clamp
- Euglycemic hyperinsulinemic clamp
- Hyperglycemic hyperinsulinemic clamp

2.2.7.1.1 Hypoglycemic Hyperinsulinemic Clamp

Plasma glucose level was decreased gradually and held at approximately 40 mg/dl to 50 mg/dl by administrating a continuous infusion of insulin (1mU/kg/min) and a variable infusion of glucose. The hypoglycemic clamp is the gold standard to investigate the hypoglycemic counter regulatory response (22).

2.2.7.1.2 Euglycemic Hyperinsulinemic Clamp

Concentration of plasma glucose was held constantly at approximately 80 mg/dl to 100 mg/dl by using a continuous infusion of insulin (1mU/kg/min) and by a variable glucose infusion using the negative feedback principle.
If euglycemic conditions are prevailing it means that the glucose infusion rate equals glucose uptake by all body tissues. Therefore, it is a measurement of tissue sensitivity to exogenous insulin (13). The euglycemic clamp is the gold standard to investigate insulin sensitivity, glucose uptake and production (23).

2.2.7.1.3 Hyperglycemic Hyperinsulinemic Clamp

Plasma glucose concentration was raised up to 125 mg/dl above basal levels (100 mg/dl) by infusion of glucose. The required hyperglycemic level was maintained by a variable glucose infusion rate (GIR) based on the negative feedback principle.
By holding the plasma-glucose constant, GIR can be used for indexing glucose metabolism (13).

2.2.7.2 Oxygen Clamp

In this study, blood pO_2 variation of the animal was essential to be able to test influence of oxygenation changes on the sensor. Therefore, we developed a method to be capable of changing pO_2 levels without harming the animal. We achieved that by adjusting the oxygen supply of the ventilator machine and then analyzing the blood gases, in order to check whether the current pO_2 value is desired one. After a couple of repetitions of this procedures it was possible to adjust the animals' pO_2 very precisely.

The pO_2 clamp is a not yet standardized procedure to manipulate the blood oxygenation. Therefore, there is no literature available.

2.2.7.3 Clamp Protocol

In order to be able to evaluate if the porcine model has the capacity to test glucose-sensors with regard to stability and accuracy it was essential to perform three different clamp protocols. The experiments were subdivided into three experiment protocols (A, B and C) each applying different profiles of blood glucose levels and blood oxygenation levels.

2.2.7.3.1 Clamp Protocol A

In clamp protocol A, the aim was to verify the sensors' responses to a dynamically changing glucose curve with disregard to blood oxygen concentrations (see Figure 8). 5 animals underwent the clamp protocol A according to Figure 8. Each experiment consisted of two 5-hour cycles, each composed of 3 glycaemic periods: normoglycemia (100 mg/dl), hypoglycemia (40 mg/dl) and hyperglycemia (200 mg/dl; induced by a variable glucose infusion) according to the clamp procedure described in section 2.2.7.1. Blood oxygenation was measured but not regulated.

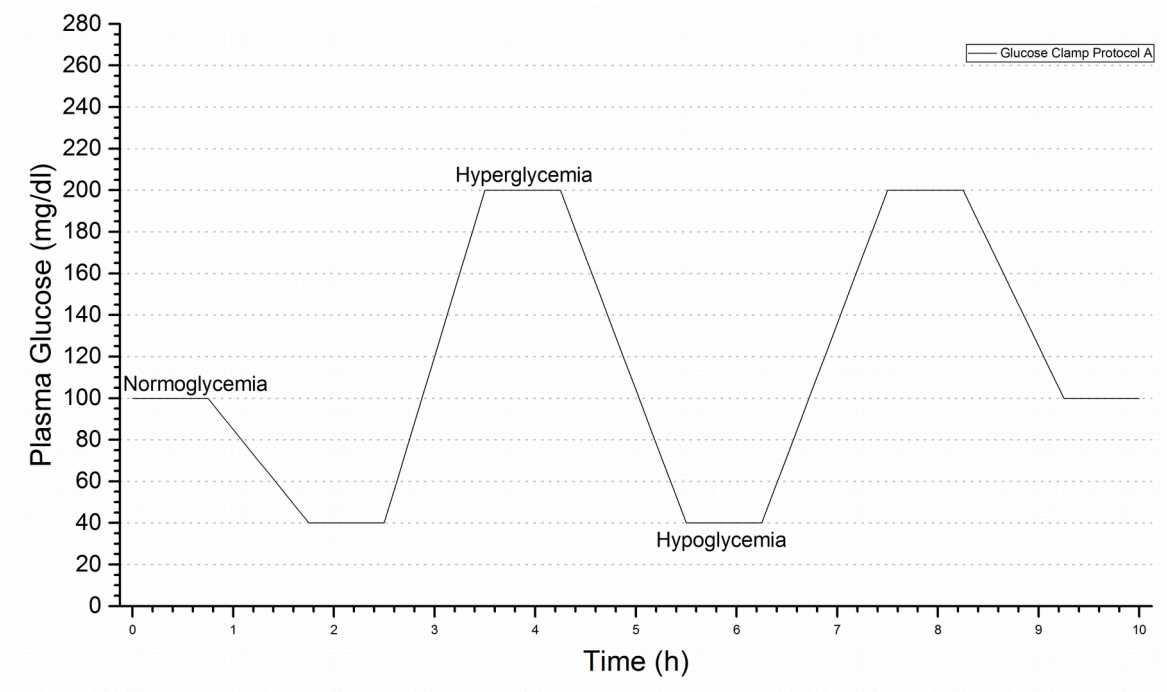

Figure 8 Clamp protocol A

2.2.7.3.2 Clamp Protocol B

In clamp protocol B less variation in plasma glucose compared to part A were planed. However, in this protocol pO_2 changings were evaluated during variable glucose concentrations (see Figure 9).

4 animals underwent the clamp protocol B according to Figure 9. Glucose was kept normoglycemic (100mg/dl) for a period of 2 hours, thereafter a 2,5 hour hypoglycemic (40mg/dl) period was followed by a 2.5 hour hyperglycemic (200 mg/dl) period. For the remaining 3 hours, glycaemia was kept at a normoglycemic (100 mg/dl) range again. In addition to the glycaemic excursions, arterial oxygen concentration was variable. For the first two hours, arterial oxygenation was increased from 100mmHg to 200mmHg. This was achieved through increase of oxygen content in inhaled air of the pig. The veterinarian performed the changes. During the following 5-hour hypo- and hyperglycemic period, blood oxygen was kept at a physiological level. This was necessary to be able to exclude the influence of oxygenation on the sensors' glucose signals. During the remaining three hours blood oxygen was stepwise decreased to 60mmHg (1 hour) and 40mmHg (1hour) and finally kept at physiological level of approximately 100mmHg.

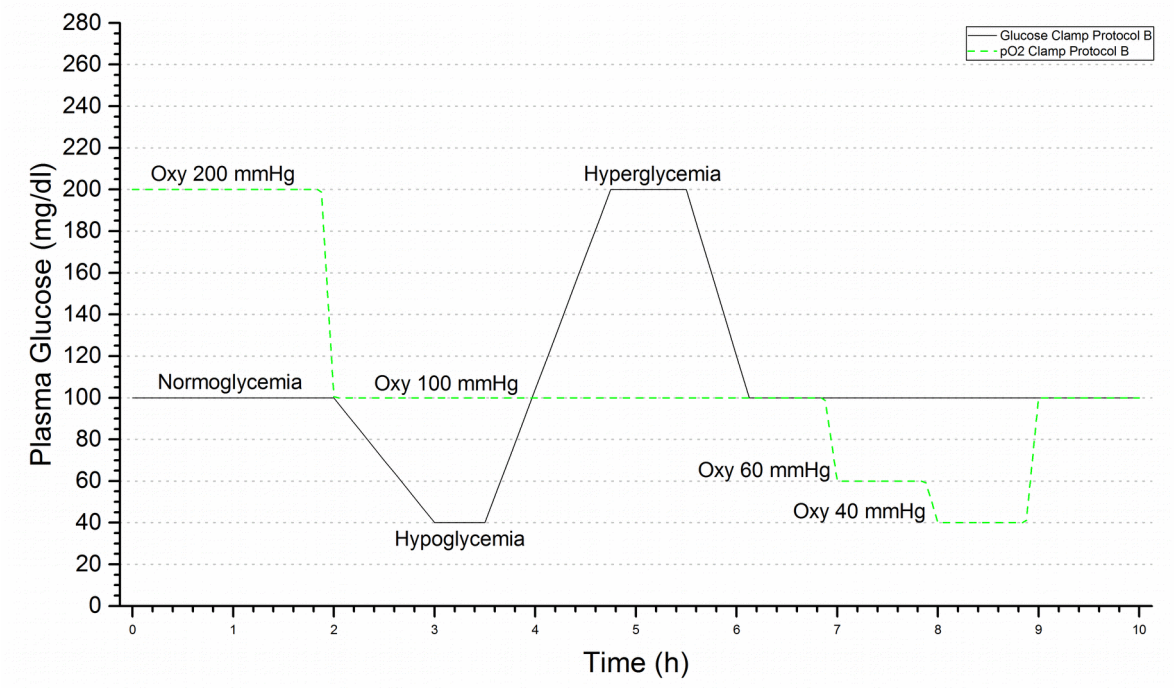

Figure 9 Clamp protocol B

2.2.7.3.3 Clamp Protocol C

This clamp protocol was performed only once. Intention of this part was very similar to clamp protocol B but this time just focusing on the variation of pO_2 and its influence on the sensors with disregard to glucose excursions. The glucose levels were held constantly in a normoglycemic range (see Figure 10).

During this experiment glucose was kept normoglycemic (100 mg/dl) for 10 hours. For the first two hours, arterial oxygenation was increased from 100mmHg to 200mmHg. Then the blood oxygen was stepwise decreased 150mmHg for 2 hours, 100 mmHg (2 hours), 60 mmHg (2 hours) and 40mmHg (1 hour) and finally kept at physiological level of approximately 100mmHg.

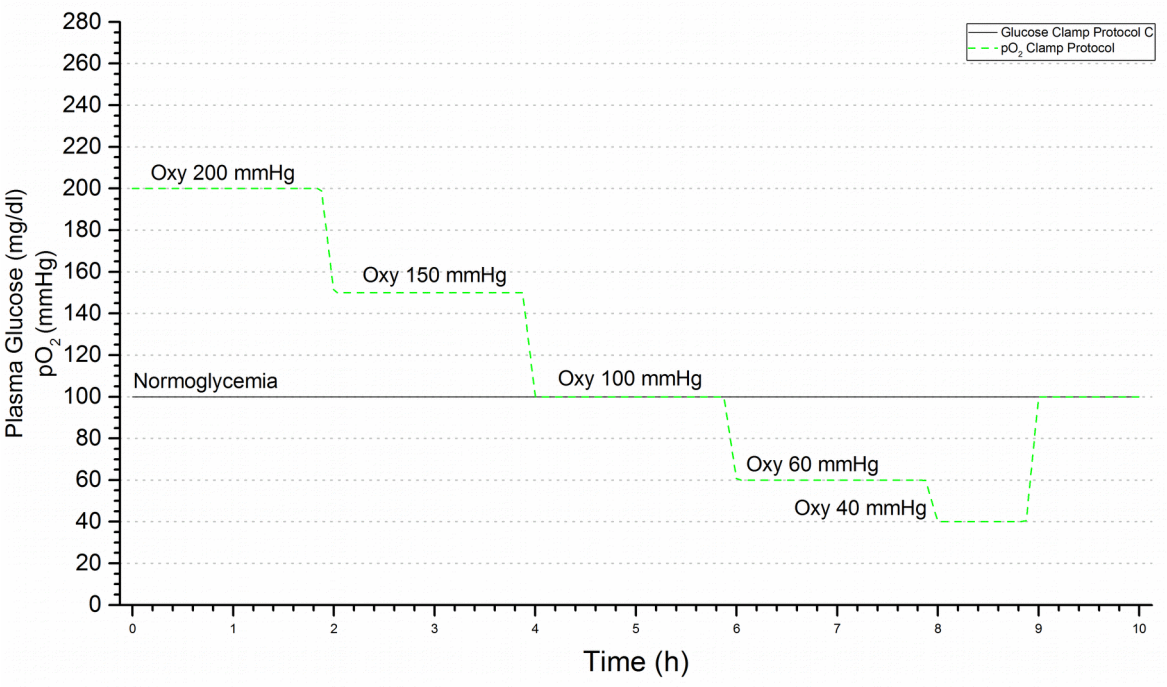

Figure 10 Clamp protocol C

2.2.8 End of the Experiments

At the end of each experiment, the veterinarian sacrificed the pigs using a potassium chloride injection.

2.3 Data Collection

All collected data was documented in a standardized case report form (CRF). Each animal had its own CRF.

The CRFs contained following information:

- Animal number.
- Date of the experiment.
- Type of experiment (e.g. A, B or C)
- Information for completion and correction using the good clinical practice (GCP) guidelines.
- Insulin infusion calculations.
- Glucose values.
- pO_2 values.
- GIR values.
- Comments.
- BGA printouts.
- Documentation of adverse events (AE).

2.4 Statistical Analysis

2.4.1 Hypothesis

The present animal model is a reproducible and feasible model for investigating glucose sensors.

2.4.2 Statistical Methods

The clamp results and time profiles of plasma glucose concentrations as well as the safety parameters were characterized using descriptive statistical methods (mean, standard deviation or standard error of the mean).

The glycemic variability during the glucose clamps was evaluated using the coefficient of variation. It represents the mean intra-individual variability within the particular hypo- or hyperglycemia period (calculated as (SD/mean) * 100).

3 Results

3.1 Compliance with the study protocol

Adherence to the study protocol was high without deviations throughout the conduction of the trial.

3.2 Animals

All animals completed the 10 hour experiments.

3.2.1 Baseline characteristics

Baseline characteristics of all pigs included in the trial are displayed in Table 1. The health status of all animals was checked by a veterinary a few days prior to the experiment.

Subject Number	1	2	3	4	5	6	7	8	9	10
Age (month)	3	3	3	3	3	3	3	2	2	3
Weight (kg)	41.5	36.8	44.1	32.9	33.2	33.7	44	31.9	29.3	38,1
Sex (m/f)	f	m	m	m	f	m	f	f	m	f
Temperature (°C)	37.7	37.3	37	36.1	36.6	37.5	37	38.5	37.5	37.6
Type of anesthesy	comb.inh.	comb.inh.	comb.inh.	comb.inh.	comb.inh.	comb.inh.	comb.inh.	comb.inh.	comb.inh.	comb.inh.

Table 1 Baseline characteristics of the animals

Mean ± standard deviation (SD): mean age was 2.8 ± 0.42 months, mean weight 36.55 ± 5.23 kg and mean body temperature 37.28 ± 0.66 °C. Half of the investigated animals were females.

3.3 Anesthesia

3.3.1 Safety Parameters

Intensive monitoring was assured using a central venous catheter, 2 arterial lines (intra-arterial blood pressure), suprapubic urine catheter (fluid balance), pulse oximetry on the tail stub, esophageal temperature sensor and ECG.

The average 11-hour narcosis was generally well tolerated. Mean values of particular vital signs throughout the experiment are summarized in Table 2.

VITAL SIGNS	Protocol A	Protocol B	Protocol C
	Mean ± SD	Mean ± SD	Mean ± SD
Systolic blood pressure (mmHg)	104.1 ± 12.9	98.7 ± 14.4	89.2 ± 14.8
Diastolic blood pressure (mmHg)	52.9 ± 6.6	49.8 ± 10.4	44.2 ± 10.7
Heart rate [beats per min]	85.5 ± 14.8	102.3 ± 36.1	91.7 ± 14.5
O2 saturation (%)	98.2 ± 3.8	95.9 ± 9.7	94.4 ± 7.4
Body temperature (°C)	37.8 ± 1.3	37.8 ± 0.8	37.9 ± 0.9
Fluid intake (l)	5.0 ± 0.2	4.6 ± 0.1	4,5
Fluid output (l)	1.8 ± 0.9	2.3 ± 0.8	3
Fluid balance (l)	+ 3.2 ± 0.9	+ 2.3 ± 0.9	+ 1.5

Table 2 Vital signs during the experiments

Ionic balance was checked hourly along with blood gas analysis and potassium was continuously supplemented according to results.

3.3.2 Medication

General anesthesia was introduced via inhalation Sevoflurane gas (1.5-1.8%).

Following medication was used continuously for maintenance of general anesthesia and stability of the animals' vital parameters:

- Analgetics:
    - Fentanyl, continuous infusion.
    - Tramadol (Tramabene®), bolus if needed.

- Methadone (Heptadon®), bolus if needed.

- Myorelaxants:
  - Cis-Atracurium (Nimbex®), bolus if needed.
  - Rocuronium (Esmeron®), bolus if needed.
  - Pancuronium, bolus if needed.
- Intravenous Fluids
  - Ringer's solution.
  - Saline solution 0.9%, for line flushing.
  - Potassium supplementation, continuous infusion.
  - Heparin, continuous infusion.
  - Insulin (Novorapid®), variable infusion and bolus if needed.
  - Glucose 20%, variable infusion and bolus if needed.

3.3.3 Heparin

Except of the first 3 animals, heparin was added into the drain saline solution of the arterial lines in each experiment because of blood clot formation in the biosensors and arterial lines during the first 3 experiments; it was a suggestion from the veterinary to prevent coagulation and to reduce the impact of the frequent blood aspirations. The amount of infused heparin is summarized in Table 3.

The amount of infused heparin cannot be compared with human heparin administration because heparin infusion is adjusted in dependence of the activated partial thromboplastin time (aPTT) value. Since the available human analytics are not applicable for porcine blood the coagulation parameter aPTT was not assessed in the present experiment.

Subject No.	Amount of heparin (I.U./h)
1	none
2	none
3	none
4	4520
5	2260
6	2500
7	2500

8	2500
9	2500
10	2500

Table 3 Amount of infused heparin

Mean ± (SD): mean amount of infused heparin in for all animals was 1928.00 ± 1476.34 I.U. The mean amount only for animals which got heparin was 2754.29 ± 783.73 I.U.

3.3.4 Adverse Events and Reactions

Table 4 summarizes the adverse events that occurred during the experiment along with their probable cause, as judged by anesthetist, and applied treatment.

COMPLICATION	No. of cases	CAUSE	TREATMENT
Cardiovascular instability	1	Individual variability	No treatment, continuous monitoring
Continuous tachycardia	1	Individual variability	No treatment, continuous monitoring
Misplaced tracheal Tubus	1	Displacement of tracheal tubus	Tracheotomy,intubation
No Urine	2	Misplaced urinary catheter	No treatment, continuous monitoring
Hypothermia	2	Cause is questionabele, a cold day?	Thermal insulation
Continuous tachycardia	1	Individual variability	No treatment, continuous monitoring
Misplaced tracheal Tubus	1	Displacement of tracheal tubus	Tracheotomy, intubation
No Urine	2	Misplaced urinary catheter	No treatment, continuous monitoring

Table 4 Overview of adverse events occurred during the experiments

All adverse events have been successfully treated and all animals were able to complete the 10-hour sampling protocol.

3.4 Clamp results

3.4.1 Clamp Protocol A

3.4.1.1 Mean

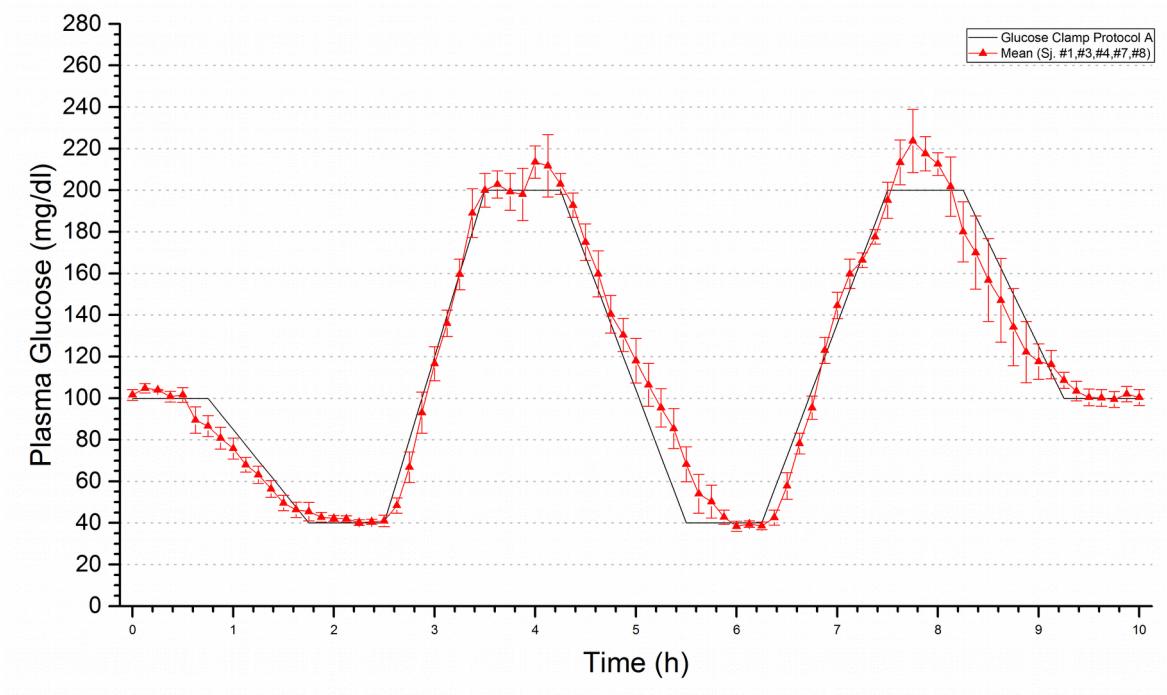

Figure 11 Mean time profile of subjects' #1, #3, #4, #7, #8 plasma glucose concentrations during the experiments

Target (mg/dl)	Coefficient of Variation				
	Subject #1	Subject #3	Subject #4	Subject #7	Subject #8
100	17.41%	7.75%	6.22%	9.07%	4.28%
40	15.20%	10.15%	6.45%	6.58%	12.39%
200	6.32%	15.70%	7.06%	7.44%	2.64%
40	18.98%	32.27%	28.13%	34.28%	32.06%
200	24.26%	5.32%	4.24%	6.76%	10.03%
100	3.46%	7.67%	8.95%	7.92%	7.33%

Table 5 Coefficient of variation (CV) in glucose target

The mean CV of glucose target 100 mg/dl was 8.01%±3.78%, the mean CV of glucose target 40 mg/dl 19.65%±11.09% and the mean CV of glucose target 200 mg/dl was 8.98%±6.45%.

3.4.1.2 Subject #1

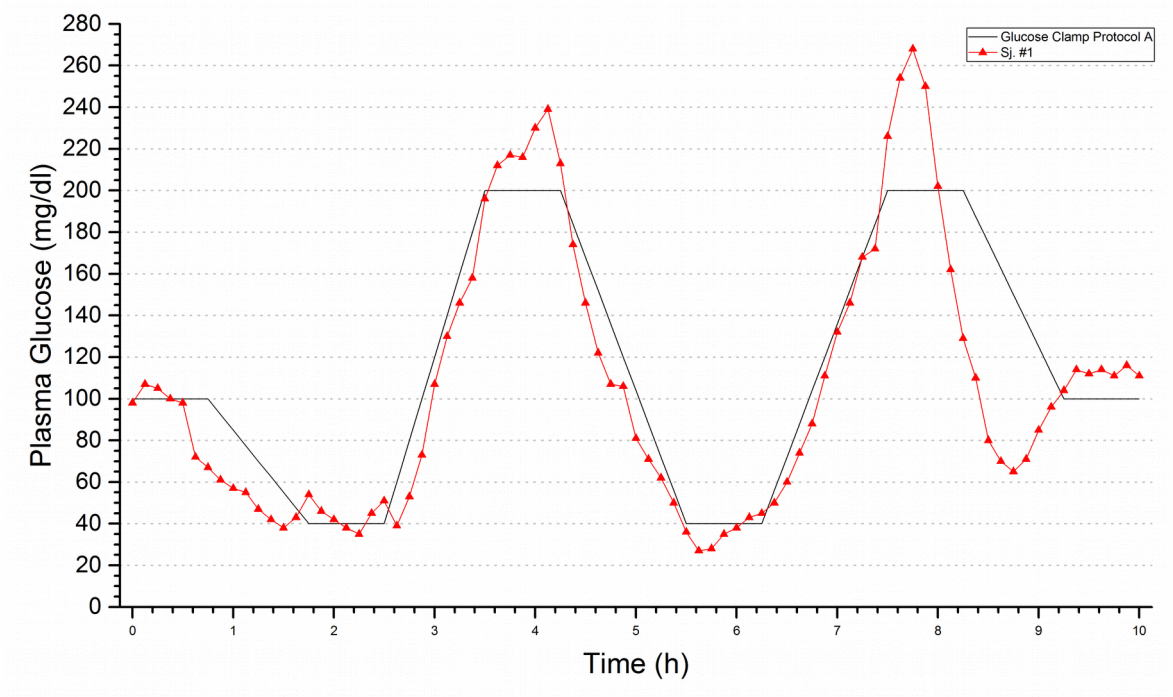

Figure 12 Time profile of subject's #1 plasma glucose concentrations during the experiment

3.4.1.3 Subject #3

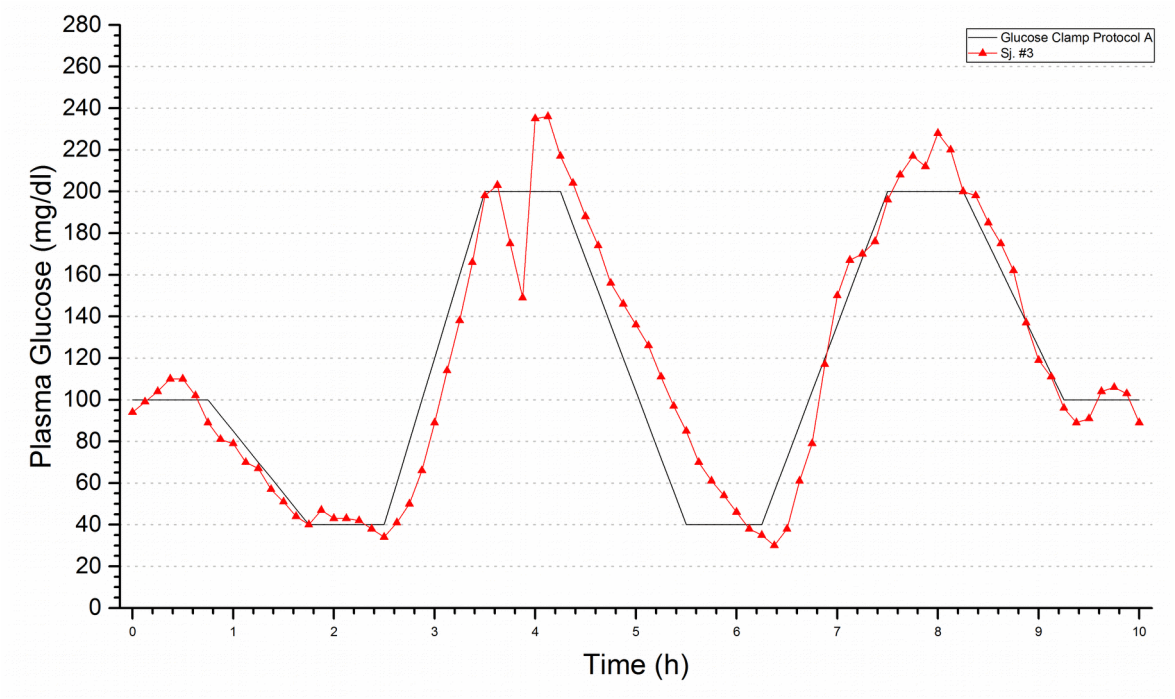

Figure 13 Time profile of subject's #3 plasma glucose concentrations during the experiment

3.4.1.4 Subject #4

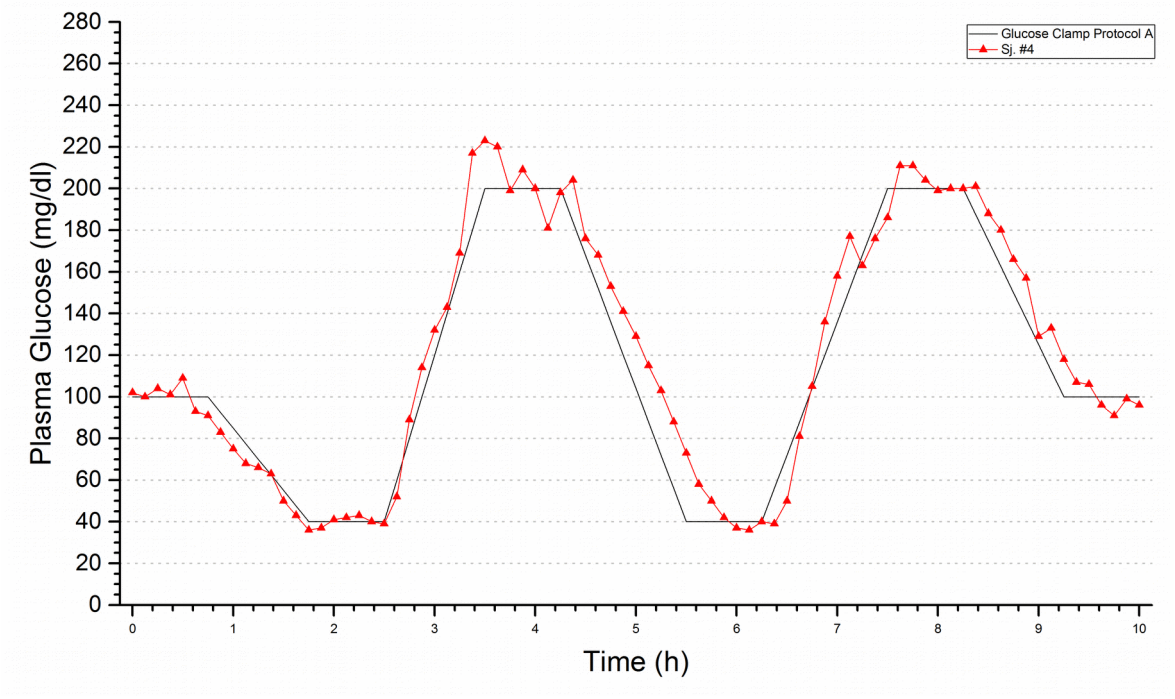

Figure 14 Time profile of subject's #4 plasma glucose concentrations during the experiment

3.4.1.5 Subject 7

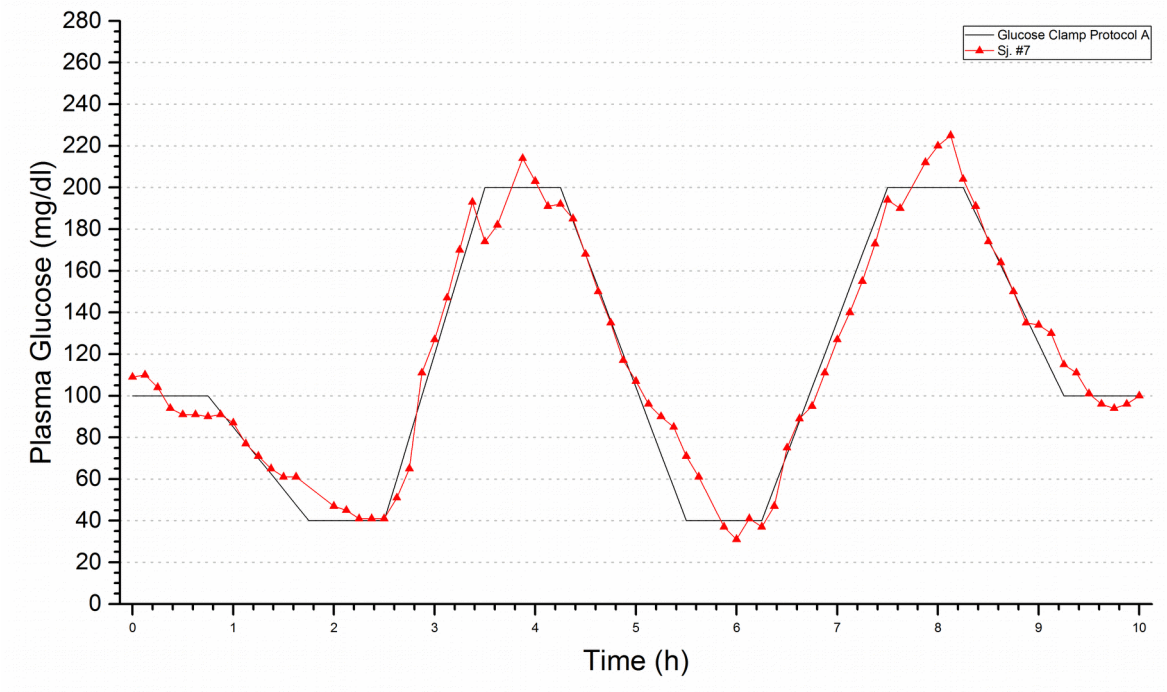

Figure 15 Time profile of subject's #7 plasma glucose concentrations during the experiment

3.4.1.6 Subject #8

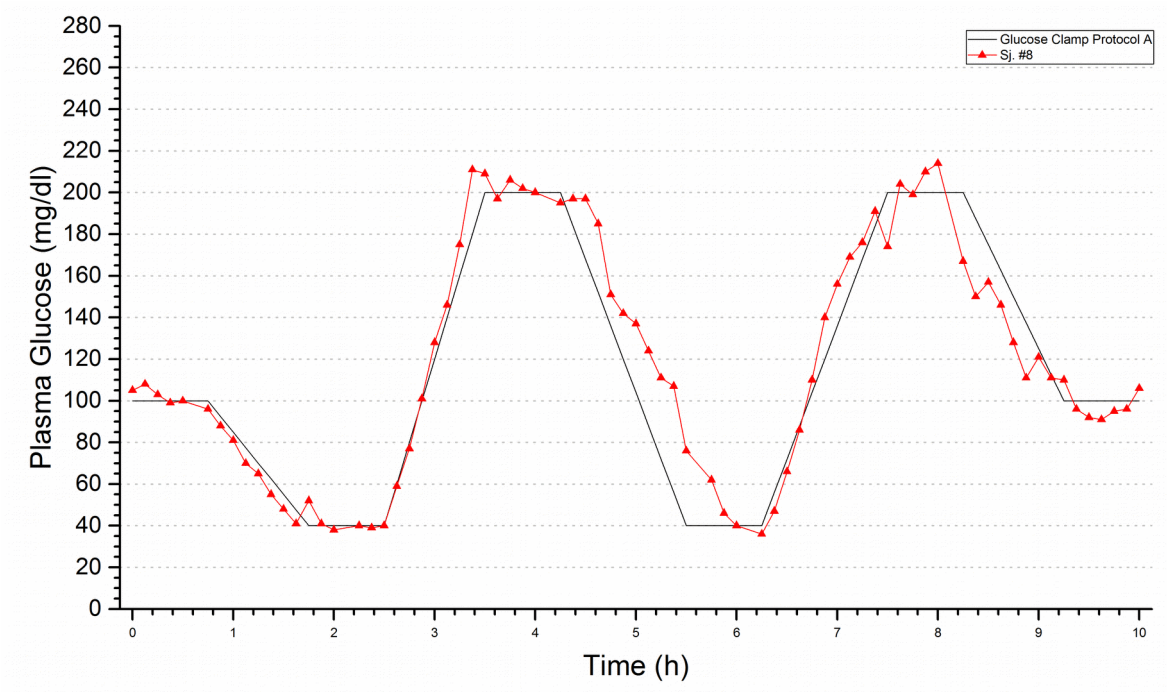

Figure 16 Time profile of subject's #8 plasma glucose concentrations during the experiment

3.4.2 Clamp Protocol B

3.4.2.1 Mean

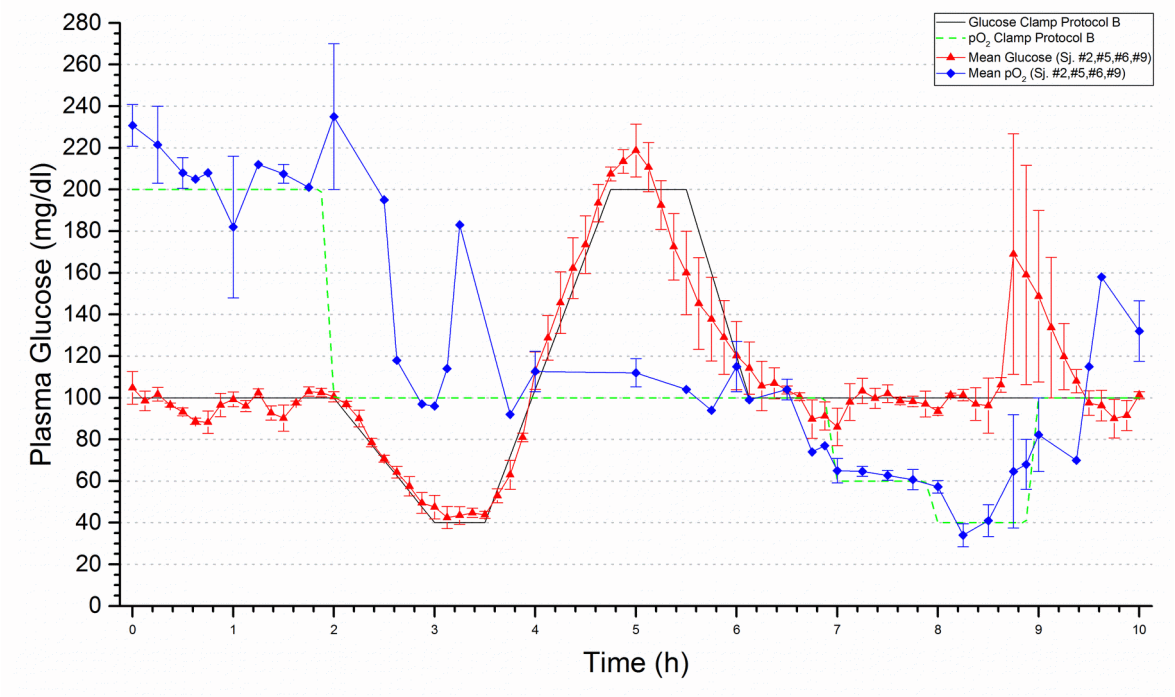

Figure 17 Mean time profile of subjects' #2,#5,#6,#9 plasma glucose and blood oxygen concentrations during the experiments

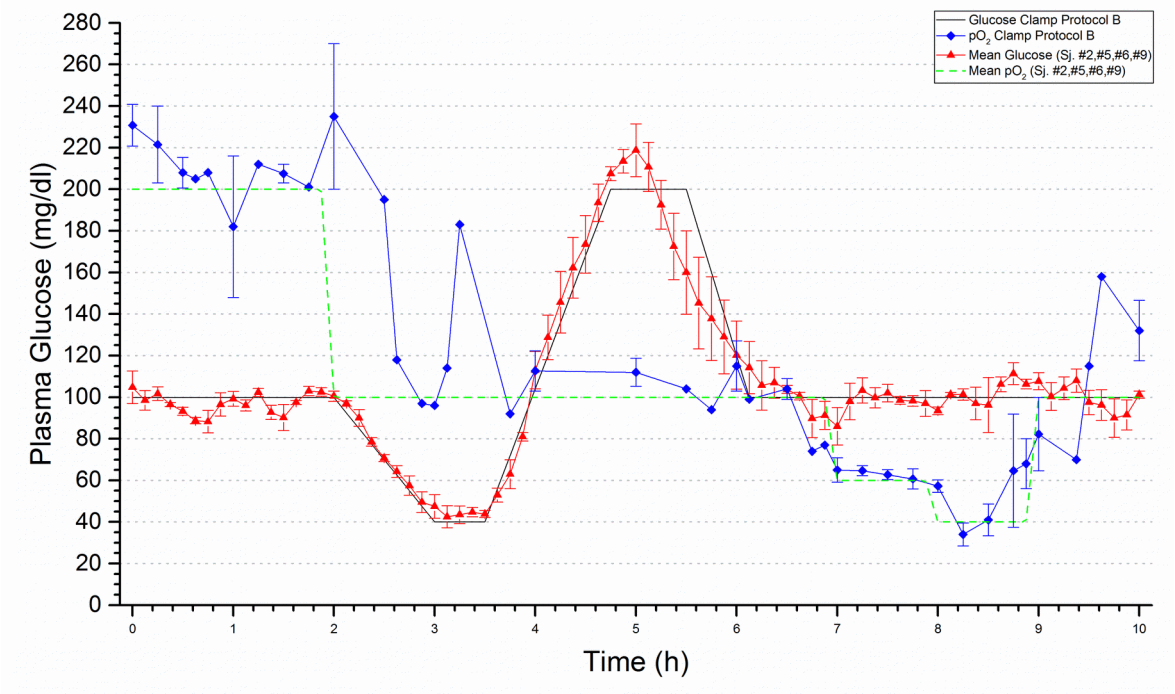

Figure 18 Mean time profile of subjects' #2,#5,#6,#9 plasma glucose and blood oxygen concentrations during the experiments - outlier excluded

In Figure 18 the outliers were excluded to smooth the graph. The high values in subject #6 were because of failure of the insulin line.

Target (mg/dl)	Coefficient of Variation			
	Subject #2	Subject #5	Subject #6	Subject #9
100	5.65%	7.14%	12.23%	5.70%
40	10.21%	5.06%	8.65%	10.79%
200	3.91%	13.71%	26.38%	8.93%
100	10.61%	7.59%	63.61%	7.99%

Table 6 Coefficient of variation in glucose target

The mean CV of glucose target 100 mg/dl was 15.06%±19.75%, the mean CV of glucose target 40 mg/dl 8.68%±2.58% and the mean CV of glucose target 200 mg/dl was 13.23%±9.63%.

3.4.2.2 Subject #2

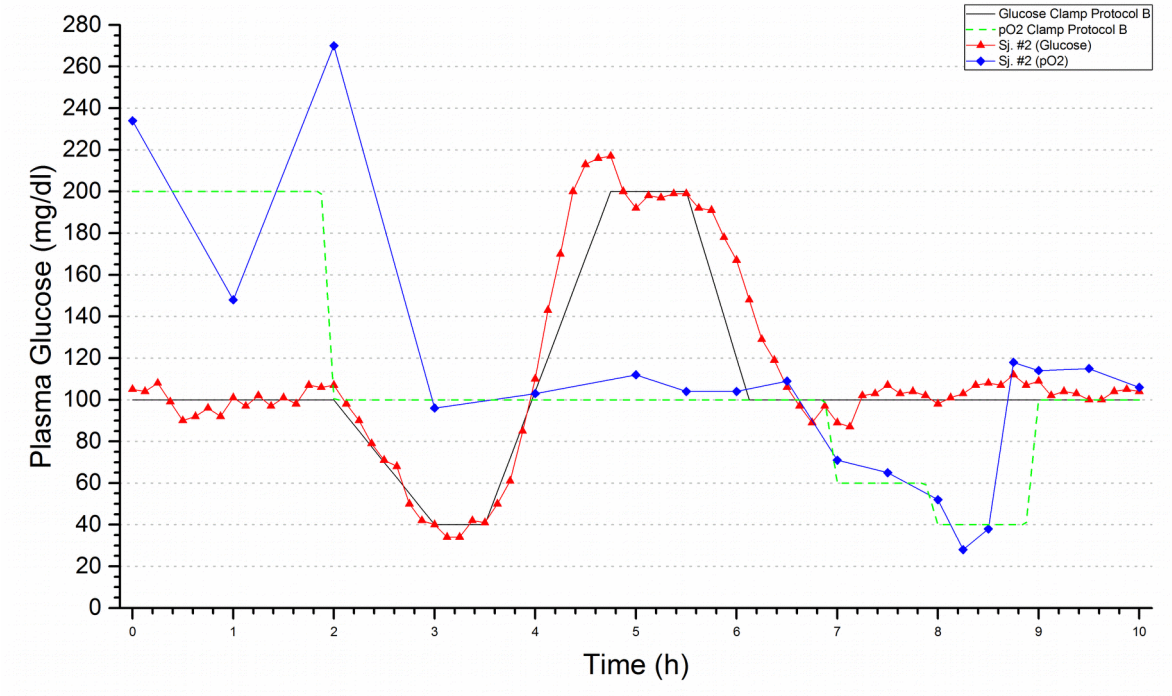

Figure 19 Time profile of subject's #2 plasma glucose and blood oxygen concentrations during the experiment

3.4.2.3 Subject #5

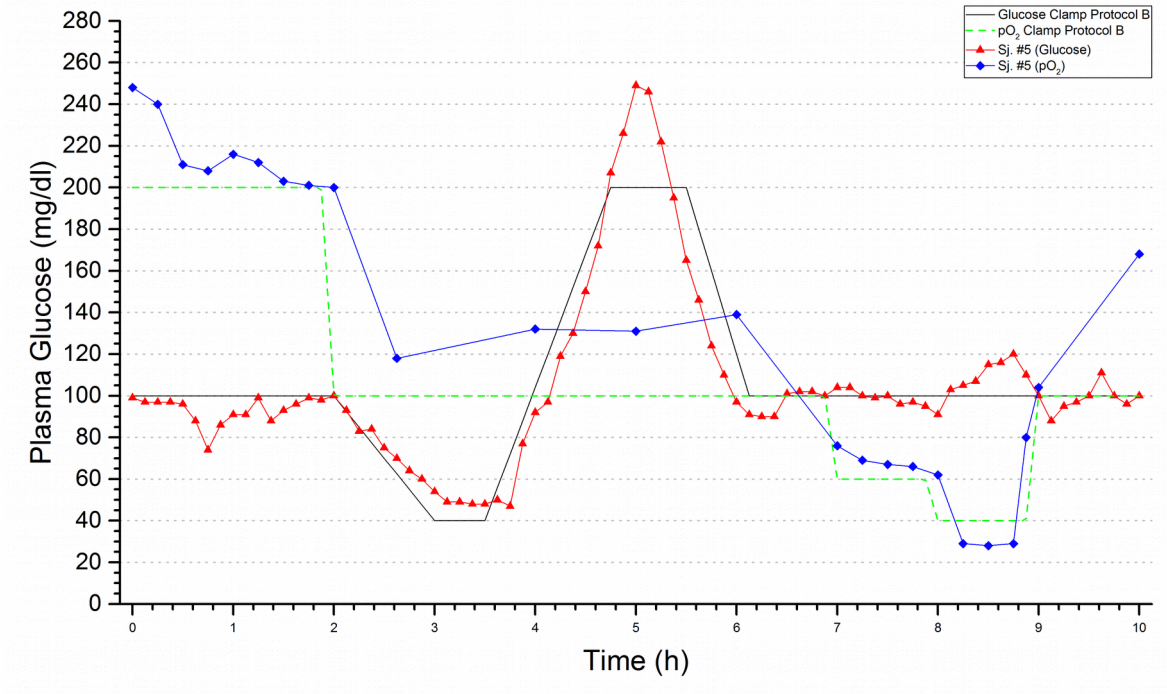

Figure 20 Time profile of subject's #5 plasma glucose and blood oxygen concentrations during the experiment

3.4.2.4 Subject #6

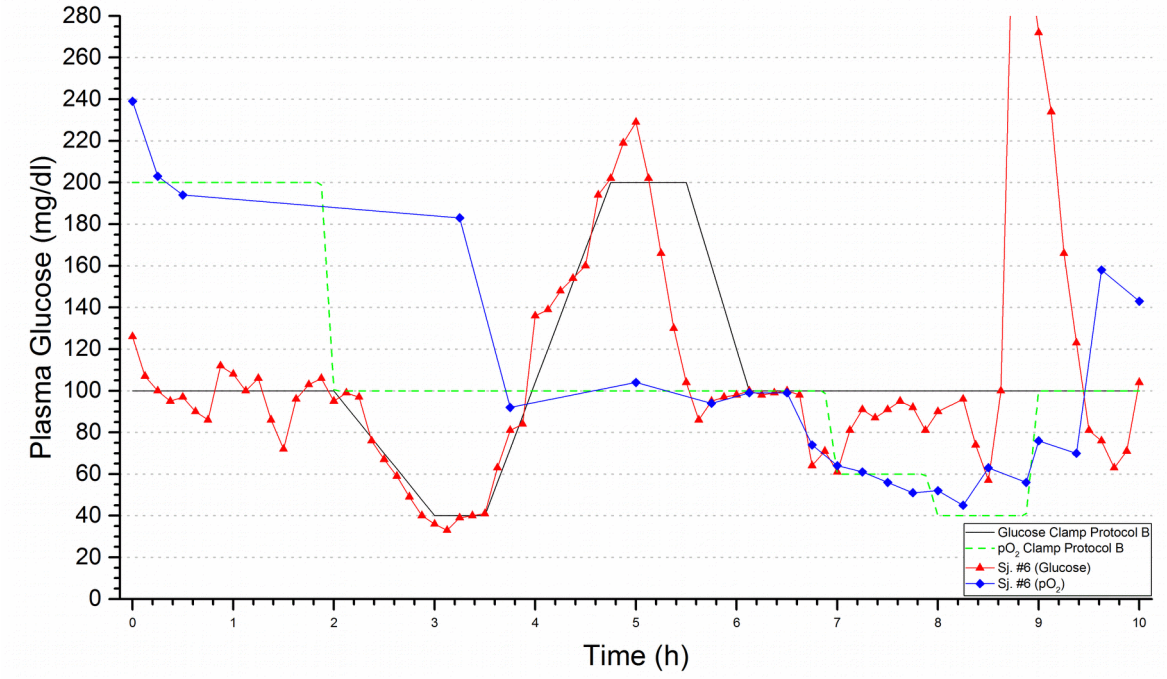

Figure 21 Time profile of subject's #6 plasma glucose and blood oxygen concentrations during the experiment

3.4.2.5 Subject #9

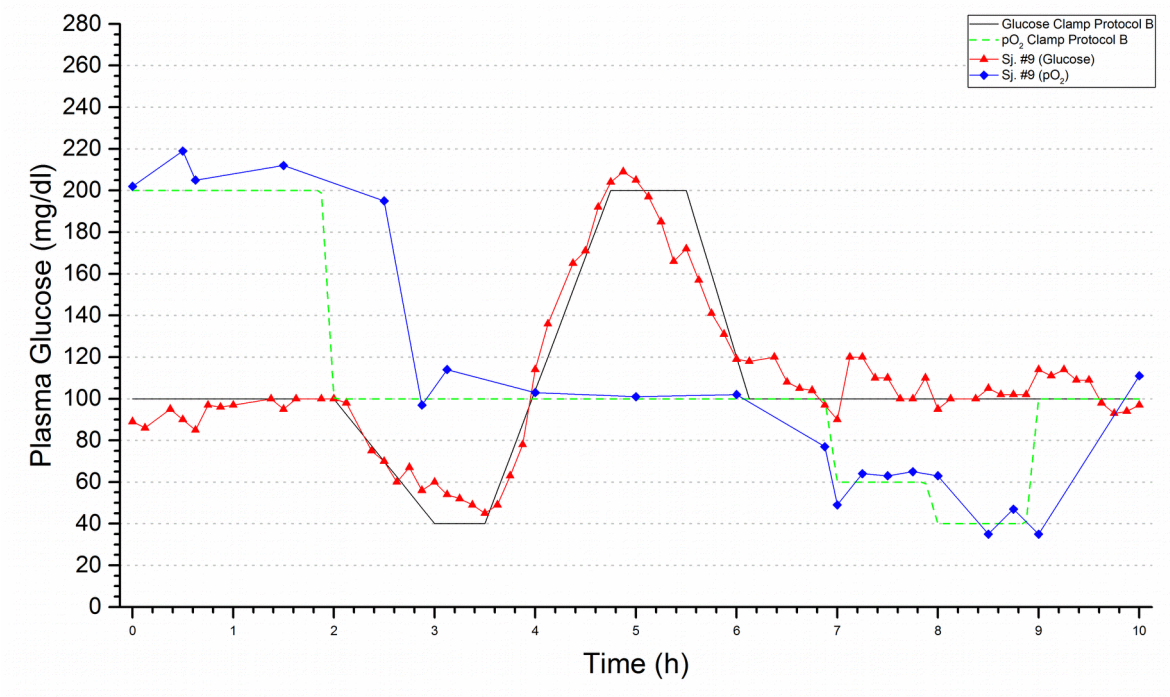

Figure 22 Time profile of subject's #9 plasma glucose and blood oxygen concentrations during the experiment

3.4.3 Clamp Protocol C

Subject #10

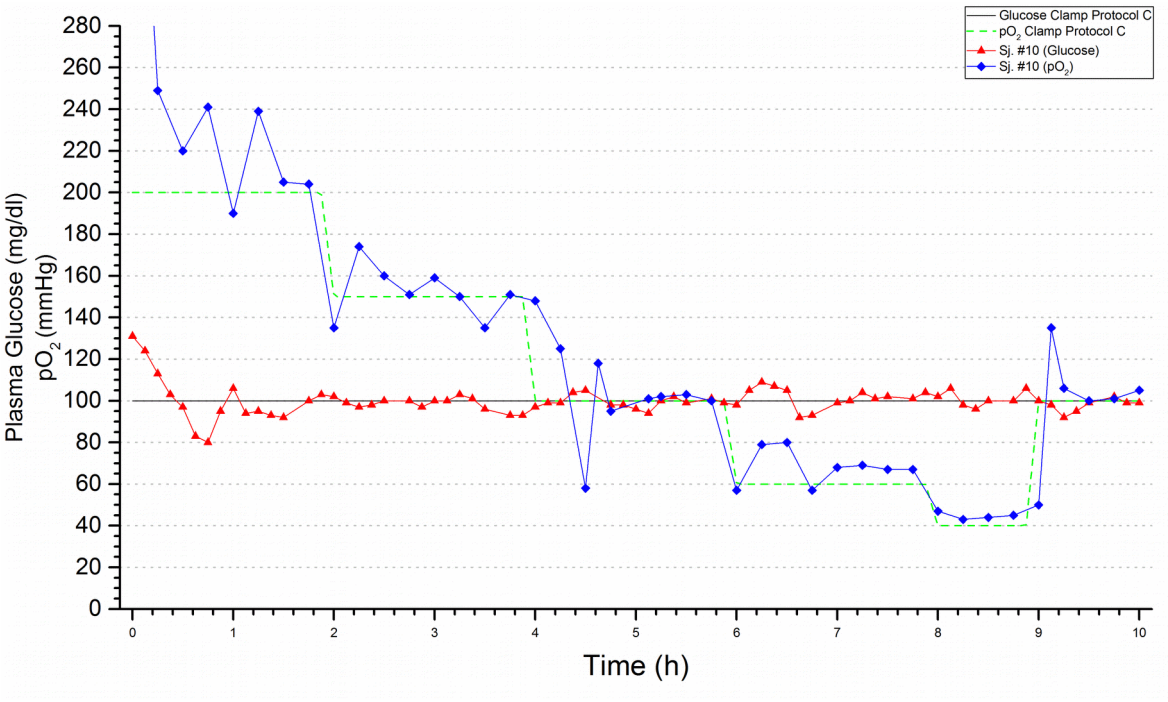

Figure 23 Time profile of subject's #10 plasma glucose and blood oxygen concentrations during the experiment

The mean CV for clamp protocol C during the 10 hour normoglycemic period was 6.99%.

4 Discussion

4.1 General Feasibility

The present thesis has shown the feasibility and reproducibility of the porcine model as an animal model which allows investigating the accuracy of biosensors with regard to variable glucose and oxygen concentrations.

4.2 Animals

According to the baseline characteristics, the animals were comparable with regard to age, temperature and type of anaesthesia. The animals differed in sex and weight. The lightest pork was 29.3 kg (Sj. #9) and the heaviest 44 kg (Sj. #6). Although there was a great difference, it should not have influenced the results because the medication, glucose infusion and insulin infusion were calculated depending on the animals' weight. Nevertheless, it can not be assured for 100 percent whether and in what extent the difference of weight and sex did not influence - even if only partial – the outcomes of this thesis

4.3 Clamps

4.3.1 Glucose Clamps

To be able to describe the quality of a glucose clamp a specific parameter is needed. One parameter usually used for this purpose is the CV. The CV is an indicator for the precision of the clamp; this means how strong the data points vary in a given period (24). Clamps are regarded to have a sufficient quality when the CV is below 5% (25). A limitation of the CV as a parameter for glucose clamp quality is that it does not describe the whole experiment. It is just a summation of all data points in a given range and time period. Critical parts of the clamp are not displayed in the CV. As done in this thesis it is possible to evaluate different

phases of the experiments separately in order to show if certain phases were harder to fulfil.

Compared to other studies the mean CVs in the present thesis were higher (13). Nevertheless, the objective of the clamps was to produce a variation of glucose and oxygen values for a specified time period and to follow given clamp curves. As the figures in the results section show, this was achieved. However, the glucose and oxygen clamps can still be improved for future experiments.

There are four different possible factors, that could have potentially influenced the experiments and consequently also the outcome of the CVs:

- Experience of the clamp-staff
- Number of staff members involved in the clamp performance
- Lack of clamp-experience in animals
- Anaesthesia of the animals

The clamp experience of the clamp-staff performing the different clamps described in this thesis differed widely. The clamp team was composed of three persons with a minimum of one-year clamp experience and high frequency of performance of clamps in humans, two persons with a minimum of one-year clamp experience but low frequency of performance of clamps in human and one trainee. So in total, a number of six persons were involved in performing the clamp on the animals, which was probably too high. During each 10 hours experiment two to three different persons performed the clamps, meaning that even during a single experiment the experience of the clamp staff varied. This could explain why during the same experiment the CVs vary as shown in the result section.
Another influencing factor could have been the presence of a trainee at each experiment. Typically, the trainee would initially propose a change of insulin or glucose rates at each sampling interval, needing a confirmation and therefore additional time of the observer to confirm the proposals of the trainee. The time spent for these decisions, in a 7.5 minutes sampling interval, was probably missing for the observer's decision building.

Furthermore, our clamp staff was not experienced to clamp pigs and needed to get used to the metabolic reaction patterns of the pigs. The fact that we clamped animals for the first time could have influenced the outcomes.
Finally, the anaesthesia itself could have influenced the outcome. Anaesthesia and its medication are a physical distress for the animals and their bodies, this means that different physiological mechanism could make the glucose clamp more difficult than in awake human subjects.
With the information above, one could argue, that the CVs during the whole experiments should have ameliorated towards the end, which they did not. This is probably related to the fact that not all of the staff members performing the clamps, performed the same number of clamps, leading to a large variability in the amelioration of adaption from human to animal.

Another explanation for the poor CV values achieved in the experiments could be that it was hard to stabilize blood glucose immediately after the dynamic clamp periods of increase and decrease. The sampling time schedule was very tight and very often the blood glucose reached the required blood glucose range too late. This certainly influenced the CVs although the mean clamp curves look quite good.

For one experiment, it also needs to be mentioned, that in this specific experiment blood glucose was held for 10 hours in a steady state but it was not possible to achieve a CV below 5%. Maybe only one single experiment was to less for a reasonable statistical evaluation but it showed that even under this conditions it was hard for the clamp team to achieve a qualitative CV value.

Apart from this CV evaluation, it is important to stress that we managed to provide the glucose biosensors with the requested values of different intended ranges as the curves in the results section and the tables in the appendix show (see point 3.4 and 7).

4.3.2 Oxygen Clamps

The oxygen clamps had too less data points for a reasonable statistical evaluation. This should be taken into consideration for future experiments. Nevertheless, it fulfilled its function to provide the glucose biosensors with different blood oxygen levels.

5 Conclusion

This thesis indicates that the anesthetized porcine model is capable to create a safe and effective preclinical environment in which it is possible to evaluate biosensors during different glucose and oxygen ranges.

Nevertheless, the following recommendations and limitations for this animal model can be drawn out of our observations during this thesis:

- The presence of an anesthetist is necessary for the whole duration of the experiment. In order to ensure effective and timely treatment of possible adverse reactions careful anesthesiology supervision is of great importance. Each adverse event needs an appropriate treatment.

- The clamp staff should be experienced in animal clamp procedures and trainees should be trained in experiments with a less tight time schedule.

- The filigree inguinal arteries of the pig with limited possibilities of cannula re-application might be a limitation. Blood vessels of pigs are more fragile than human blood vessels because the wall-thickness is smaller and so the risk of rupture is much bigger. Although in none of the experiments such a problem occurred, it should be taken into consideration because it is a possible reason for abort of an experiment.

- The state of health of the pigs can't be compared with the ones of patients on ICUs, where some of the tested glucose biosensors are intended for future use. Critically ill patients with respiratory insufficiency can have chemical and physiological reactions, which may affect the biosensor.

- In our setting the animals were sacrificed after the experiments, so it was not possible to perform a second experiment with the same animal in order evaluate reproducibility of the whole glucose clamp procedure.

- Another limitation of the present experiments was the fact, that our setting with its 10-hour observation time did not reflect the real time biosensors are planned to be applied in humans. Therefore, for future experiments it might be useful to investigate if another animal model can be applied for an extended period of time.

6 References

1. van den Berghe G, Wouters P, Weekers F, Verwaest C, Bruyninckx F, Schetz M, et al. Intensive insulin therapy in critically ill patients. N Engl J Med. 2001 Nov 8;345(19):1359–67.
2. Scheen AJ. [Clinical study of the month. Toxicity of hyperglycemia in intensive care and beneficial effects of optimized insulin therapy]. Rev Médicale Liège. 2001 Dec;56(12):859–61.
3. Johan Groeneveld AB, Beishuizen A, Visser FC. Insulin: a wonder drug in the critically ill? Crit Care Lond Engl. 2002 Apr;6(2):102–5.
4. Binnekade JM. Intensive insulin treatment reduced mortality and morbidity in critically ill patients. Evid Based Nurs. 2002 Apr;5(2):53.
5. Ingels C, Debaveye Y, Milants I, Buelens E, Peeraer A, Devriendt Y, et al. Strict blood glucose control with insulin during intensive care after cardiac surgery: impact on 4-years survival, dependency on medical care, and quality-of-life. Eur Heart J. 2006 Nov;27(22):2716–24.
6. Lazakidou AA. Web-Based Applications in Healthcare and Biomedicine. Springer Science & Business Media; 2009. 276 p.
7. John E. Hall. Guyton and Hall Textbook of Medical Physiology, 12e. 12th edition. Philadelphia, Pa: Saunders; 2010. p. 464.
8. Boutrand J-P. Biocompatibility and Performance of Medical Devices. Elsevier; 2012. 557 p.
9. Siller-Matula JM, Plasenzotti R, Spiel A, Quehenberger P, Jilma B. Interspecies differences in coagulation profile. Thromb Haemost. 2008 Sep;100(3):397–404.
10. Wilcox G. Insulin and Insulin Resistance. Clin Biochem Rev. 2005 May;26(2):19–39.
11. Espinal J. Understanding Insulin Action: Principles and Molecular Mechanisms. Springer Science & Business Media; 2013. p. 17.
12. Ellahham S. Insulin therapy in critically ill patients. Vasc Health Risk Manag. 2010;6:1089–101.
13. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol. 1979 Sep;237(3):E214–

23.
14. Ledoux J, Werner ME, Brayden JE, Nelson MT. Calcium-Activated Potassium Channels and the Regulation of Vascular Tone. Physiology. 2006 Feb 1;21(1):69–78.
15. Terker AS, Zhang C, McCormick JA, Lazelle RA, Zhang C, Meermeier NP, et al. Potassium Modulates Electrolyte Balance and Blood Pressure through Effects on Distal Cell Voltage and Chloride. Cell Metab. 2015 Jan 6;21(1):39–50.
16. Hamm LL, Nakhoul N, Hering-Smith KS. Acid-Base Homeostasis. Clin J Am Soc Nephrol. 2015 Dec 7;10(12):2232–42.
17. Johnson RJ, Feehally J, Floege J. Comprehensive Clinical Nephrology. Elsevier Health Sciences; 2014. 1323 p.
18. DeFronzo RA, Felig P, Ferrannini E, Wahren J. Effect of graded doses of insulin on splanchnic and peripheral potassium metabolism in man. Am J Physiol. 1980 May;238(5):E421–7.
19. Nguyen TQ, Maalouf NM, Sakhaee K, Moe OW. Comparison of Insulin Action on Glucose versus Potassium Uptake in Humans. Clin J Am Soc Nephrol CJASN. 2011 Jul;6(7):1533–9.
20. Mader JK, Birngruber T, Korsatko S, Deller S, Köhler G, Boysen S, et al. Enhanced absorption of insulin aspart as the result of a dispersed injection strategy tested in a randomized trial in type 1 diabetic patients. Diabetes Care. 2013 Apr;36(4):780–5.
21. Korsatko S, Glettler K, Olsen KJ, Wutte A, Bock G, Koehler G, et al. A direct comparison of the pharmacodynamic properties of insulin detemir and neutral protamine lispro insulin in patients with type 1 diabetes. Diabetes Obes Metab. 2013 Mar;15(3):241–5.
22. Pieber TR, Deller S, Korsatko S, Jensen L, Christiansen E, Madsen J, et al. Counter-regulatory hormone responses to hypoglycaemia in people with type 1 diabetes after 4 weeks of treatment with liraglutide adjunct to insulin: a randomized, placebo-controlled, double-blind, crossover trial. Diabetes Obes Metab. 2015 Aug;17(8):742–50.
23. Conn PM. Animal Models for the Study of Human Disease. 1st ed. London ; Waltham, MA: Academic Press; 2013. 1089 p.
24. Benesch C, Heise T, Klein O, Heinemann L, Arnolds S. How to Assess the Quality of Glucose Clamps? Evaluation of Clamps Performed With ClampArt, a

Novel Automated Clamp Device. J Diabetes Sci Technol. 2015 Jul 1;9(4):792–800.
25. Heinemann L, Ampudia-Blasco FJ. Glucose clamps with the Biostator: a critical reappraisal. Horm Metab Res Horm Stoffwechselforschung Horm Métabolisme. 1994 Dec;26(12):579–83.

7 Appendix

7.1 Glucose Targets

Based on the figures of glucose protocol A,B and C glucose targets were defined in Table 7.

Time	Targets Protocol A	Targets Protocol B	Targets Protocol C
00:00:00	100	100	100
00:07:30	100	100	100
00:15:00	100	100	100
00:22:30	100	100	100
00:30:00	100	100	100
00:37:30	100	100	100
00:45:00	100	100	100
00:52:30		100	100
01:00:00		100	100
01:07:30		100	100
01:15:00		100	100
01:22:30		100	100
01:30:00		100	100
01:37:30		100	100
01:45:00	40	100	100
01:52:30	40	100	100
02:00:00	40	100	100
02:07:30	40		100
02:15:00	40		100
02:22:30	40		100
02:30:00	40		100
02:37:30			100
02:45:00			100
02:52:30			100
03:00:00		40	100

03:07:30		40	100
03:15:00		40	100
03:22:30		40	100
03:30:00	200	40	100
03:37:30	200		100
03:45:00	200		100
03:52:30	200		100
04:00:00	200		100
04:07:30	200		100
04:15:00	200		100
04:22:30			100
04:30:00			100
04:37:30			100
04:45:00		200	100
04:52:30		200	100
05:00:00		200	100
05:07:30		200	100
05:15:00		200	100
05:22:30		200	100
05:30:00	40	200	100
05:37:30	40		100
05:45:00	40		100
05:52:30	40		100
06:00:00	40		100
06:07:30	40	100	100
06:15:00	40	100	100
06:22:30		100	100
06:30:00		100	100
06:37:30		100	100
06:45:00		100	100
06:52:30		100	100
07:00:00		100	100
07:07:30		100	100

07:15:00		100	100
07:22:30		100	100
07:30:00	200	100	100
07:37:30	200	100	100
07:45:00	200	100	100
07:52:30	200	100	100
08:00:00	200	100	100
08:07:30	200	100	100
08:15:00	200	100	100
08:22:30		100	100
08:30:00		100	100
08:37:30		100	100
08:45:00		100	100
08:52:30		100	100
09:00:00		100	100
09:07:30		100	100
09:15:00	100	100	100
09:22:30	100	100	100
09:30:00	100	100	100
09:37:30	100	100	100
09:45:00	100	100	100
09:52:30	100	100	100
10:00:00	100	100	100

Table 7 Glucose targets for glucose protocol A,B and C

7.2 Glucose Targets Results

In the following tables the blue column shows the steady state areas. The red cells indicate missing values.

7.2.1 Glucose Targets Protocol A

Time (hh:mm:ss)	Target (mg/dl)	Subject #1	Subject #3	Subject #4	Subject #7	Subject #8
00:00:00	100	98	94	102	109	105
00:07:30	100	107	99	100	110	108
00:15:00	100	105	104	104	104	103
00:22:30	100	100	110	101	94	99
00:30:00	100	98	110	109	91	100
00:37:30	100	72	102	93	91	
00:45:00	100	67	89	91	90	96
01:45:00	40	54	40	36		52
01:52:30	40	46	47	37		41
02:00:00	40	42	43	41	47	38
02:07:30	40	38	43	42	45	
02:15:00	40	35	42	43	41	40
02:22:30	40	45	38	40	41	39
02:30:00	40	51	34	39	41	40
03:30:00	200	196	198	223	174	209
03:37:30	200	212	203	220	182	197
03:45:00	200	217	175	199		206
03:52:30	200	216	149	209	214	202
04:00:00	200	230	235	200	203	200
04:07:30	200	239	236	181	191	
04:15:00	200	213	217	198	192	195
05:30:00	40	36	85	73	71	76
05:37:30	40	27	70	58	61	
05:45:00	40	28	61	50		62

05:52:30	40	35	54	42	37	46
06:00:00	40	38	46	37	31	40
06:07:30	40	43	38	36	41	
06:15:00	40	45	35	40	37	36
07:30:00	200	226	196	186	194	174
07:37:30	200	254	208	211	190	204
07:45:00	200	268	217	211		199
07:52:30	200	250	212	204	212	210
08:00:00	200	202	228	199	220	214
08:07:30	200	162	220	200	225	
08:15:00	200	129	200	200	204	167
09:15:00	100	104	96	118	115	110
09:22:30	100	114	89	107	111	96
09:30:00	100	112	91	106	101	92
09:37:30	100	114	104	96	96	91
09:45:00	100	111	106	91	94	95
09:52:30	100	116	103	99	96	96
10:00:00	100	111	89	96	100	106

Table 8 Results glucose targets protocol A

7.2.2 Deviation from Target Protocol A

Time (hh:mm:ss)	Target (mg/dl)	Subject #1	Subject #3	Subject #4	Subject #7	Subject #8
00:00:00	100	2%	6%	2%	9%	5%
00:07:30	100	7%	1%	0%	10%	8%
00:15:00	100	5%	4%	4%	4%	3%
00:22:30	100	0%	10%	1%	6%	1%
00:30:00	100	2%	10%	9%	9%	0%
00:37:30	100	28%	2%	7%	9%	
00:45:00	100	33%	11%	9%	10%	4%
01:45:00	40	35%	0%	10%		30%
01:52:30	40	15%	18%	8%		3%
02:00:00	40	5%	8%	3%	18%	5%
02:07:30	40	5%	8%	5%	13%	
02:15:00	40	13%	5%	8%	3%	0%
02:22:30	40	13%	5%	0%	3%	3%
02:30:00	40	28%	15%	3%	3%	0%
03:30:00	200	2%	1%	12%	13%	5%
03:37:30	200	6%	2%	10%	9%	2%
03:45:00	200	9%	13%	1%		3%
03:52:30	200	8%	26%	5%	7%	1%
04:00:00	200	15%	18%	0%	2%	0%
04:07:30	200	20%	18%	10%	5%	
04:15:00	200	7%	9%	1%	4%	3%
05:30:00	40	10%	113%	83%	78%	90%
05:37:30	40	33%	75%	45%	53%	
05:45:00	40	30%	53%	25%		55%
05:52:30	40	13%	35%	5%	8%	15%
06:00:00	40	5%	15%	8%	23%	0%
06:07:30	40	8%	5%	10%	3%	
06:15:00	40	13%	13%	0%	8%	10%
07:30:00	200	13%	2%	7%	3%	13%

07:37:30	200	27%	4%	6%	5%	2%
07:45:00	200	34%	9%	6%		1%
07:52:30	200	25%	6%	2%	6%	5%
08:00:00	200	1%	14%	1%	10%	7%
08:07:30	200	19%	10%	0%	13%	
08:15:00	200	36%	0%	0%	2%	17%
09:15:00	100	4%	4%	18%	15%	10%
09:22:30	100	14%	11%	7%	11%	4%
09:30:00	100	12%	9%	6%	1%	8%
09:37:30	100	14%	4%	4%	4%	9%
09:45:00	100	11%	6%	9%	6%	5%
09:52:30	100	16%	3%	1%	4%	4%
10:00:00	100	11%	11%	4%	0%	6%

Table 9 Deviation from target protocol A

7.2.3 Glucose Targets Protocol B

Time (hh:mm:ss)	Target (mg/dl)	Subject #2	Subject #5	Subject #6	Subject #9
00:00:00	100	98	94	102	109
00:07:30	100	104	97	107	86
00:15:00	100	108	97	100	
00:22:30	100	99	97	95	95
00:30:00	100	90	96	97	90
00:37:30	100	92	88	90	85
00:45:00	100	96	74	86	97
00:52:30	100	92	86	112	96
01:00:00	100	101	91	108	97
01:07:30	100	97	91	100	
01:15:00	100	102	99	106	
01:22:30	100	97	88	86	100
01:30:00	100	101	93	72	95
01:37:30	100	98	96	96	100
01:45:00	100	107	99	103	
01:52:30	100	106	98	106	100
02:00:00	100	107	100	95	100
03:00:00	40	40	54	36	60
03:07:30	40	34	49	33	54
03:15:00	40	34	49	39	52
03:22:30	40	42	48	40	49
03:30:00	40	41	48	41	45
04:45:00	200	217	207	202	204
04:52:30	200	200	226	219	209
05:00:00	200	192	249	229	205
05:07:30	200	198	246	202	197
05:15:00	200	197	222	166	185
05:22:30	200	199	195	130	166
05:30:00	200	199	165	104	172
06:07:30	100	148	91	100	118
06:15:00	100	129	90	98	
06:22:30	100	119	90	99	120
06:30:00	100	106	101	100	108
06:37:30	100	97	102	98	105
06:45:00	100	89	102	64	104
06:52:30	100	97	100	71	97
07:00:00	100	89	104	61	90
07:07:30	100	87	104	81	120

07:15:00	100	102	100	91	120
07:22:30	100	103	99	87	110
07:30:00	100	107	100	91	110
07:37:30	100	103	96	95	100
07:45:00	100	104	97	92	100
07:52:30	100	102	95	81	110
08:00:00	100	98	91	90	95
08:07:30	100	101	103		100
08:15:00	100	103	105	96	
08:22:30	100	107	107	74	100
08:30:00	100	108	115	57	105
08:37:30	100	107	116	100	102
08:45:00	100	112	120	342	102
08:52:30	100	107	110	317	102
09:00:00	100	109	100	272	114
09:07:30	100	102	88	234	111
09:15:00	100	104	95	166	114
09:22:30	100	103	97	123	109
09:30:00	100	100	100	81	109
09:37:30	100	100	111	76	98
09:45:00	100	104	100	63	93
09:52:30	100	105	96	71	94
10:00:00	100	104	100	104	97

Table 10 Results glucose targets protocol B

7.2.4 Deviation from Target Protocol B

Time (hh:mm:ss)	Target (mg/dl)	Subject #2	Subject #5	Subject #6	Subject #9
00:00:00	100	2%	6%	2%	9%
00:07:30	100	4%	3%	7%	14%
00:15:00	100	8%	3%	0%	
00:22:30	100	1%	3%	5%	5%
00:30:00	100	10%	4%	3%	10%
00:37:30	100	8%	12%	10%	15%
00:45:00	100	4%	26%	14%	3%
00:52:30	100	8%	14%	12%	4%
01:00:00	100	1%	9%	8%	3%
01:07:30	100	3%	9%	0%	
01:15:00	100	2%	1%	6%	
01:22:30	100	3%	12%	14%	0%
01:30:00	100	1%	7%	28%	5%
01:37:30	100	2%	4%	4%	0%
01:45:00	100	7%	1%	3%	
01:52:30	100	6%	2%	6%	0%
02:00:00	100	7%	0%	5%	0%
03:00:00	40	0%	35%	10%	50%
03:07:30	40	15%	23%	18%	35%
03:15:00	40	15%	23%	3%	30%
03:22:30	40	5%	20%	0%	23%
03:30:00	40	3%	20%	3%	13%
04:45:00	200	9%	4%	1%	2%
04:52:30	200	0%	13%	10%	5%
05:00:00	200	4%	25%	15%	3%
05:07:30	200	1%	23%	1%	2%
05:15:00	200	2%	11%	17%	8%
05:22:30	200	1%	3%	35%	17%
05:30:00	200	1%	18%	48%	14%
06:07:30	100	48%	9%	0%	18%
06:15:00	100	29%	10%	2%	
06:22:30	100	19%	10%	1%	20%
06:30:00	100	6%	1%	0%	8%
06:37:30	100	3%	2%	2%	5%
06:45:00	100	11%	2%	36%	4%
06:52:30	100	3%	0%	29%	3%
07:00:00	100	11%	4%	39%	10%
07:07:30	100	13%	4%	19%	20%
07:15:00	100	2%	0%	9%	20%

07:22:30	100	3%	1%	13%	10%
07:30:00	100	7%	0%	9%	10%
07:37:30	100	3%	4%	5%	0%
07:45:00	100	4%	3%	8%	0%
07:52:30	100	2%	5%	19%	10%
08:00:00	100	2%	9%	10%	5%
08:07:30	100	1%	3%		0%
08:15:00	100	3%	5%	4%	
08:22:30	100	7%	7%	26%	0%
08:30:00	100	8%	15%	43%	5%
08:37:30	100	7%	16%	0%	2%
08:45:00	100	12%	20%	242%	2%
08:52:30	100	7%	10%	217%	2%
09:00:00	100	9%	0%	172%	14%
09:07:30	100	2%	12%	134%	11%
09:15:00	100	4%	5%	66%	14%
09:22:30	100	3%	3%	23%	9%
09:30:00	100	0%	0%	19%	9%
09:37:30	100	0%	11%	24%	2%
09:45:00	100	4%	0%	37%	7%
09:52:30	100	5%	4%	29%	6%
10:00:00	100	4%	0%	4%	3%

Table 11 Deviation from target protocol B

7.2.5 Glucose Targets Protocol C

Time (hh:mm:ss)	Target (mg/dl)	Subject #10	Deviation from Target	CV
00:00:00	100	131	31%	6.99%
00:07:30	100	124	24%	
00:15:00	100	113	13%	
00:22:30	100	103	3%	
00:30:00	100	97	3%	
00:37:30	100	83	17%	
00:45:00	100	80	20%	
00:52:30	100	95	5%	
01:00:00	100	106	6%	
01:07:30	100	94	6%	
01:15:00	100	95	5%	
01:22:30	100	93	7%	
01:30:00	100	92	8%	
01:37:30	100			
01:45:00	100	100	0%	
01:52:30	100	103	3%	
02:00:00	100	102	2%	
02:07:30	100	99	1%	
02:15:00	100	97	3%	
02:22:30	100	98	2%	
02:30:00	100	100	0%	
02:37:30	100			
02:45:00	100	100	0%	
02:52:30	100	97	3%	
03:00:00	100	100	0%	
03:07:30	100	100	0%	
03:15:00	100	103	3%	
03:22:30	100	101	1%	
03:30:00	100	96	4%	
03:37:30	100			
03:45:00	100	93	7%	
03:52:30	100	93	7%	
04:00:00	100	97	3%	
04:07:30	100	99	1%	
04:15:00	100	99	1%	
04:22:30	100	104	4%	
04:30:00	100	105	5%	
04:37:30	100			
04:45:00	100	98	2%	

04:52:30	100	98	2%
05:00:00	100	96	4%
05:07:30	100	94	6%
05:15:00	100	100	0%
05:22:30	100	102	2%
05:30:00	100	99	1%
05:37:30	100		
05:45:00	100	101	1%
05:52:30	100	99	1%
06:00:00	100	98	2%
06:07:30	100	105	5%
06:15:00	100	109	9%
06:22:30	100	107	7%
06:30:00	100	105	5%
06:37:30	100	92	8%
06:45:00	100	93	7%
06:52:30	100		
07:00:00	100	99	1%
07:07:30	100	100	0%
07:15:00	100	104	4%
07:22:30	100	101	1%
07:30:00	100	102	2%
07:37:30	100		
07:45:00	100	101	1%
07:52:30	100	104	4%
08:00:00	100	102	2%
08:07:30	100	106	6%
08:15:00	100	98	2%
08:22:30	100	96	4%
08:30:00	100	100	0%
08:37:30	100		
08:45:00	100	100	0%
08:52:30	100	106	6%
09:00:00	100	100	0%
09:07:30	100	98	2%
09:15:00	100	92	8%
09:22:30	100	95	5%
09:30:00	100	99	1%
09:37:30	100		
09:45:00	100	102	2%
09:52:30	100	99	1%
10:00:00	100	99	1%

Table 12 Glucose targets protocol C, deviation from target and coefficient of variation (CV)