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Effects of Low-Dose Glucagon on Subcutaneous Insulin Absorption in Pigs

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ABSTRACT

Background: Slow insulin absorption prevents the development of a fully automated artificial pancreas with subcutaneous insulin delivery.

Objective: We have hypothesized that glucagon could be used as a vasodilator to accelerate insulin absorption in a bihormonal subcutaneous artificial pancreas. The present proof-of-concept study is the first study to investigate the pharmacokinetics of insulin after subcutaneous administration of a low dose of glucagon at the site of subcutaneous insulin injection.

Methods: Twelve anesthetized pigs were randomized to receive a subcutaneous injection of 10 IU insulin aspart with either 100 µg glucagon or the equivalent volume of placebo (0.9% saline solution) injected at the same site. Arterial samples were collected for 180 minutes to determine insulin, glucagon, and glucose concentrations.

Results: Glucagon did not influence the insulin concentration T_{max} in plasma. The plasma insulin $AUC_{0-\infty}$ was significantly larger after glucagon administration ($P < 0.01$). The glucagon group had significantly higher glucose concentrations in the first 30 minutes after insulin administration ($P < 0.05$).

Conclusions: This proof-of-concept study indicates that glucagon may increase the total absorption of a single dose of subcutaneously injected insulin. This is a novel observation. However, we did not observe any reduction in insulin concentration T_{max}, as we had hypothesized. Further, glucagon induced a significant, undesirable increase in early blood glucose concentrations.

Introduction

A subcutaneous artificial pancreas; that is, a device that automatically calculates and delivers subcutaneous insulin doses based on continuous feedback from a subcutaneous glucose sensor, is a promising new treatment option for patients with diabetes mellitus type 1 (DM1). Both unihormonal devices, administering only insulin, and bihormonal devices, administering both insulin and glucagon, have been developed.[1] However, only unihormonal, hybrid devices that require meal announcements from the user are commercially available.[2] Developing a fully automated subcutaneous artificial pancreas is difficult because the device struggles with inherent delays, particularly in relation to meals. The major limiting factor is the slow absorption of insulin from subcutaneous tissue resulting in delayed effects on glucose metabolism.[3] Our research group (Artificial Pancreas Trondheim) has hypothesized using microdoses of glucagon with meal boluses of insulin in subcutaneous artificial pancreas devices to accelerate insulin absorption and conventionally sized doses to treat hypoglycemia.[4] We have shown that small doses of glucagon (10 and 100 µg) may cause a substantial increase in local subcutaneous blood flow in healthy adults.[5] We hypothesized that this effect could be utilized to accelerate insulin absorption in bihormonal subcutaneous artificial pancreata if the tubes delivering insulin and glucagon end at the same subcutaneous site.[4,6]

To substantiate our hypothesis, we performed a proof-of-concept study in anesthetized pigs to investigate possible differences in insulin pharmacokinetics following subcutaneous administration of insulin aspart with either glucagon or placebo. The

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primary aim was to investigate the T_{max} insulin concentration in plasma. C_{max} insulin concentration in plasma, $T_{1/2}$ insulin in plasma, AUC_{0-t} plasma insulin concentration, $AUC_{0-\infty}$, plasma insulin concentration, and effects on glucose metabolism were also analyzed.

Methods

Ethics approval and study design

The study was preapproved by the Norwegian Food Safety Authorities (FOTS No. 29687), and it complied with the Norwegian Regulation on the Use of Animals in Research and the 2010/63 EU directive on the Protection of Animals Used for Scientific Purposes.

We conducted an open, randomized, placebo-controlled study in anesthetized pigs during August 2022. To detect at least 10 minutes' difference in T_{max} with a power of 80% and an alpha value of 0.05, we included 6 Norwegian domestic pigs (*Sus scrofa domesticus*) in each group.

Animals and animal handling

All pigs were acquired from the same local supplier at age approximately 12 weeks. Sex was not known before randomization. Six males were included in the glucagon group, whereas 2 males and 4 females were included in the placebo group. The mean (SD) weight was 42 (7) kg in the glucagon group and 44 (8) kg in the placebo group.

The animals were monitored continuously during the experiments. All surgical procedures, drug administrations (other than premedication/light sedation), and blood samplings were performed under general anesthesia.

Study procedure

Premedication and anesthesia

The pigs were premedicated with an intramuscular injection of 10 mg/kg Azaperone (Separon vet.; Salfarm Scandinavia AS, Oslo, Norway) and 10 mg/kg Ketamine (Ketalar; Pfizer AS, Oslo, Norway), and an intravenous infusion of 1 mg atropine (Takeda AS, Asker, Norway). Anesthesia was induced by an intravenous infusion of 150 to 250 µg fentanyl (Actavis Group, Hafnarfjörður, Iceland), 75 to 125 mg thiopental (VUAB Pharma AS, Roztoky, Czech Republic), and 150 to 250 mg ketamine before intubation. Anesthesia was maintained by continuous intravenous infusion of 0.5 mg/kg/h midazolam (Accord Healthcare Limited, Middlesex, United Kingdom) and 7.5 µg/kg/h fentanyl, with continuous inhalation of 0.5% to 2% isoflurane (Baxter AS, Oslo, Norway). An intravenous infusion of 2 g Cephalothin (Cefalotin Navamedic; Navamedic ASA, Oslo, Norway) was given as antibiotic prophylaxis immediately after establishing anesthesia and repeated every third hour. The pigs were humanely killed whilst under general anesthesia at the end of the study day with an intravenous infusion of 100 mg/kg phenobarbital (NAF; Apotek, Lørenskog, Norway).

Suppression of endogenous glucagon and insulin secretion

To suppress endogenous glucagon and insulin secretion, the pigs received an initial intravenous bolus of 5 µg/kg octreotide (Sandostatin; Novartis Europharm Limited, United Kingdom) 1 hour before the study intervention, immediately followed by continuous intravenous infusion of 5 µg/kg/h octreotide throughout the study day. The concentration of porcine insulin was measured every hour to monitor the level of suppression of endogenous insulin secretion.

Insulin infusion

The blood glucose concentration was titrated to a target concentration of approximately 10 mmol/L by adjusting the intravenous infusion rate of a 20% glucose solution (Glucos B. Braun; B. Braun Melsungen AG, Germany) before insulin infusion. After this, the glucose infusion rate was kept stable to monitor the effects on glucose metabolism. However, the infusion rate was increased if the arterial glucose concentration fell below 2.8 mmol/L.

Each pig received 1 bolus of 10 IU (0.1 mL) insulin aspart (NovoRapid; Novo Nordisk AS, Denmark) together with either 100 µg (0.1 mL) glucagon reconstituted in sterile water (Novo Nordisk A/S, Denmark) or the equivalent volume of 0.9% saline solution (Natriumklorid B. Braun; B. Braun Melsungen AG).

Insulin and glucagon/placebo were administered via separate tubes by a Chemyx Fusion 100 syringe pump to the subcutaneous adipose tissue behind the left ear. Both infusions were delivered simultaneously over 60 seconds (from time 0 to time 1 minute). An insertion guide was used to ensure that the infusions were deposited at the exact same place, with a needle depth of 9 mm (Figure 1).

Blood sampling

Arterial blood samples were drawn at 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 120, 150, and 180 minutes after insulin administration. Glucose and insulin were analyzed at all time points, whereas glucagon was analyzed at 0, 10, 20, 30, 60, 120, and 180 minutes and porcine insulin at 0, 60, 120, and 180 minutes. The observation time was set to 180 minutes to cover the most important part of the postprandial period and ensure observation for minimum 2 expected half-lives of insulin aspart.[7]

Sample handling and analysis

Arterial blood glucose concentrations were analyzed in duplicate immediately after collection on a Radiometer ABL 800 FLEX blood gas analyzer. Both the intra-assay and the interassay coefficient of variation was <5%.

Insulin concentrations were measured using Iso-Insulin ELISA kits (Mercodia, Uppsala, Sweden), glucagon concentrations were measured using Glucagon ELISA kits (Mercodia), and porcine insulin concentrations were measured using Porcine Insulin ELISA kits (Mercodia). All samples were run in duplicate and performed in the same setup by the same engineer. The intra-assay and the interassay variation was <10% for glucagon and porcine insulin, and <5% for iso-insulin.

Data analysis

Pharmacokinetic analysis

C_{max} and T_{max} were obtained directly from the measured insulin concentrations in plasma. $T_{1/2}$ and AUC_{0-last} were estimated using Simbiology in MATLAB version R2020B (MathWorks, Natick, Massachusetts). The terminal rate constant (λ_z), describing the decrease of the log-concentration of insulin, was calculated by applying a best-fit linear regression to the terminal portion of the curve. $T_{1/2}$ was calculated as $\frac{\ln 2}{(\lambda z)}$. AUC_{0-t} was calculated by using the linear trapezoidal method. $AUC_{0-\infty}$ was calculated as AUC_{0-t} plus $\frac{C_{last}}{\lambda z}$, where C_{last} represents the last measured insulin concentration.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9 (GraphPad Inc, La Jolla, California). The distribution of glucose data was assessed using the D'Agostino-Pearson omnibus test and the Anderson-Darling normality test.

Medians, means and SD of T_{max}, C_{max}, AUC_{0-last}, $AUC_{0-\infty}$, and $T_{1/2}$ of insulin were calculated. Possible differences in T_{max}, C_{max},

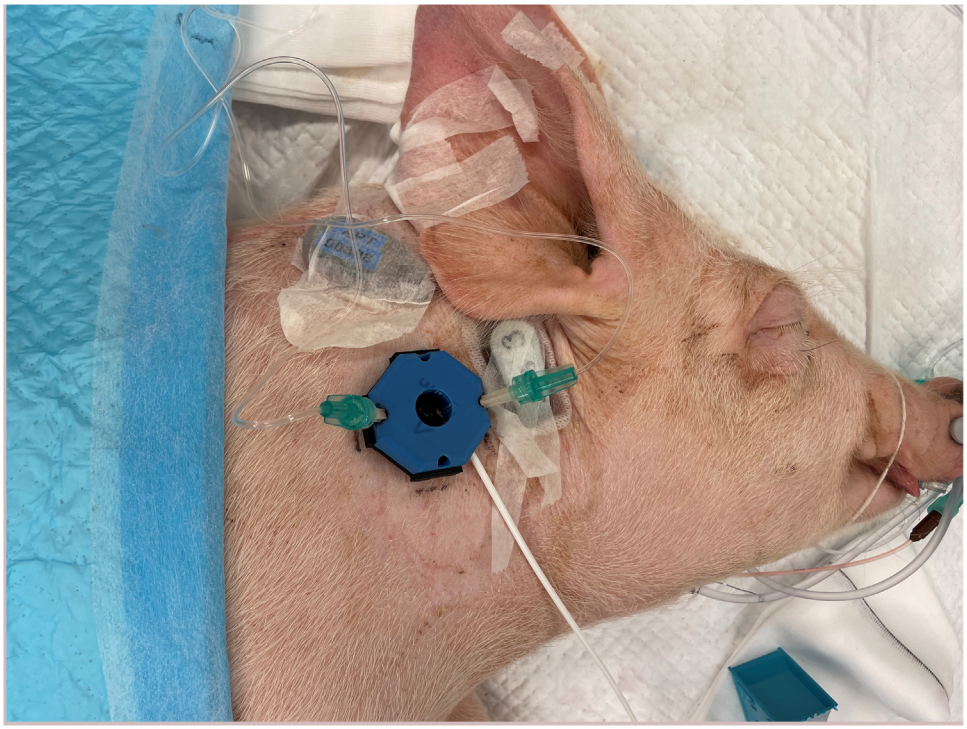

Fig. 1. Photo showing subcutaneous drug infusion on the neck of a pig. The insulin and glucagon/placebo infusions were delivered using an insertion guide (blue octagon).

AUC_{0-last}, $AUC_{0-\infty}$, and $T_{1/2}$ between the 2 groups were examined using the Mann-Whitney *U* test. Medians and means (SD) of T_{max} and $T_{1/2}$ of glucagon were calculated in the glucagon group.

Differences in glucose concentration over time between the 2 groups were analyzed using a mixed-effects analysis with time and treatment as fixed effects and the subject number as a random effect. Correction for multiple analyses was performed using Šídák's multiple comparison test.

Results

Exclusions

One pig that had been randomized to the placebo group was excluded from all pharmacokinetic and pharmacodynamic analyses because the infusions had been delivered intramuscularly. This was established through the injection of methylene blue dye after the pig was humanely killed, which was performed after all experiments to confirm drug deposition site (Figure 2).

Suppression of endogenous insulin secretion

The octreotide infusion did not fully supress endogenous insulin secretion. At time 0 (start of insulin infusion), porcine insulin was below the quantification cutoff of 2.3 mU/L in only 4 out of 12 pigs. The mean (SD) porcine insulin concentration in plasma was 7.5 (2.8) mU/L in the remaining 8 pigs.

Over the total duration of the experiments, porcine insulin was below the quantification cutoff limit in 34 of 48 samples (17 of 24 samples in both groups). In the remaining samples, the mean (SD) porcine insulin concentration was 6.8 (1.6) mU/L in the placebo group and 11.5 (7.4) in the glucagon group.

Pharmacokinetic parameters of insulin

Pharmacokinetic findings are summarized in the Table. Mean insulin concentrations over time are presented in Figure 3. The total insulin concentrations and porcine insulin concentrations over time for each individual pig in the glucagon and placebo group are presented in Figure 4 and Figure 5, respectively.

There was no significant difference between the groups when comparing T_{max}, C_{max}, $T_{1/2}$, or AUC_{0-t} between the 2 groups ($P > 0.05$ for all comparisons).

$AUC_{0-\infty}$ was significantly larger in the glucagon group ($P < 0.01$). The difference remained significant also after subtracting the AUC of porcine insulin from the total insulin data ($P < 0.01$). Median $AUC_{0-\infty}$ was 8419 mU/L/min in the glucagon group and 6211 mU/L/min in the placebo group, whereas mean (SD) $AUC_{0-\infty}$ was 8342 (961) and 6001 (689) mU/L/min in the 2 groups, respectively.

Pharmacokinetic parameters of glucagon

Endogenous glucagon secretion was efficiently supressed by octreotide because the plasma glucagon concentration was below the quantification cutoff limit of 2.18 pmol/L at time 0 in all included pigs except 1 in the placebo group. In total, the plasma glucagon concentration was below 2.18 pmol/L in 39 of 42 samples from the placebo group. In the remaining 3 samples (originating from 2 pigs) the mean (SD) glucagon concentration was 4.0 (1.5) pmol/L.

In the glucagon group, median T_{max} of glucagon was 15 minutes, and mean (SD) T_{max} was 15 (5.5) minutes. Median C_{max} of glucagon was 108 pmol/L and mean (SD) C_{max} was 107 (49) pmol/L. Median $T_{1/2}$ of glucagon was 18 minutes and mean $T_{1/2}$ (SD) was 22 (9) minutes.

Pharmacodynamic parameters

The analysis is based on the first 90 minutes of observation after the intervention because 6 pigs needed increased glucose infusions between 90 and 120 minutes because their arterial glucose concentration fell below 2.8 mmol/L. The mean change in blood glucose concentrations for both groups are presented in Figure 6.

The mean (SD) blood glucose concentration at baseline was 10.2 (0.3) mmol/L for the glucagon group and 10.2 (0.4) mmol/L for the placebo group. The glucose concentrations were corrected for baseline concentration and log-transformed to achieve normal distribution before analysis. The glucagon group had significantly higher

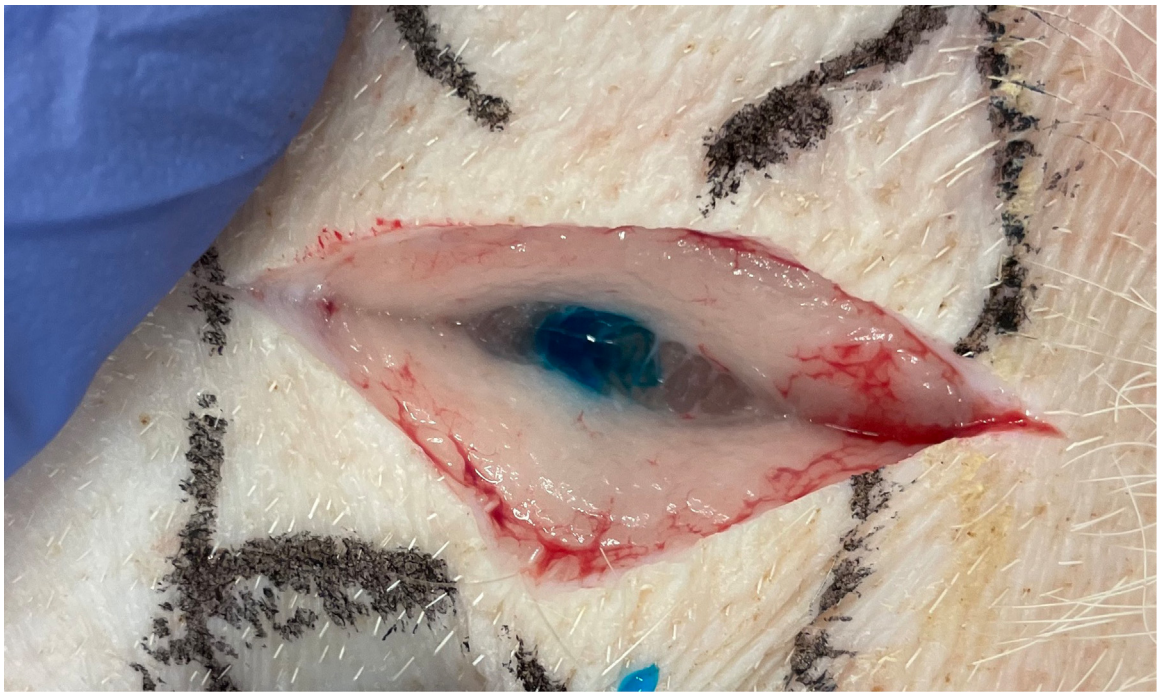

Fig 2. Photo of skin incision after injection of methylene blue dye. Tissue deposition was investigated by injecting methylene blue dye after the pigs were humanely killed at the end of all experiments. Figure 2 illustrates unintended intramuscular injection in 1 of the placebo-treated pigs.

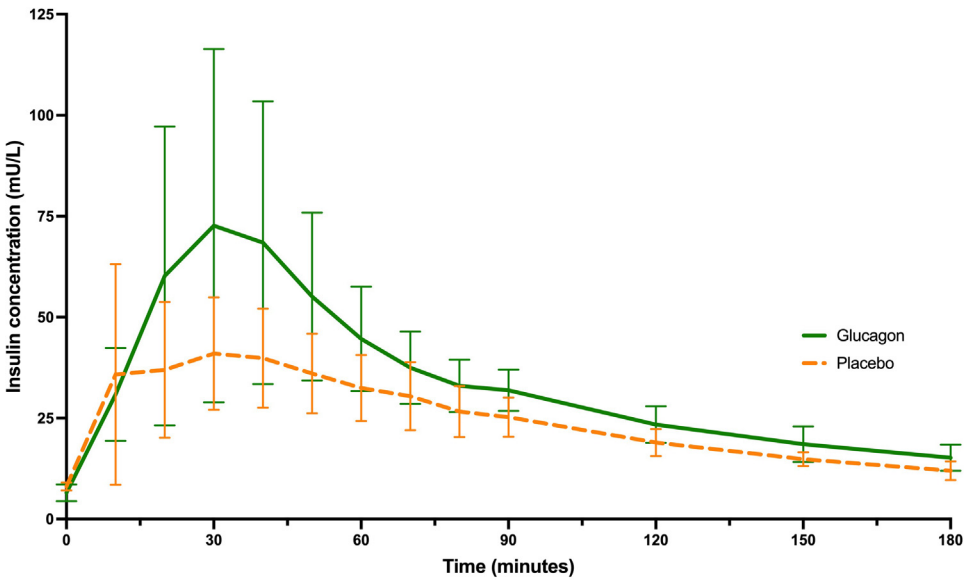

Fig 3. Mean (SD) insulin concentrations in arterial plasma over time in both study groups.

Table
Pharmacokinetic parameters of insulin.

Pharmacokinetic parameter*	Glucagon group	Placebo group
T_{max}, min	37 (10)	28 (18)
C_{max}, pmol/L	76 (42)	55 (16)
$T_{1/2}$, min	95 (39)	84 (27)
AUC_{0-t}, mU/L/min	6181 (1618)	4530 (691)
$AUC_{0-\infty}$, mU/L/min	8342 (961)	6001 (689)
$AUC_{0-\infty}$ mU/L/min with AUC of porcine insulin subtracted	7908 (1159)	5372 (994)

* Values are presented as mean (SD).

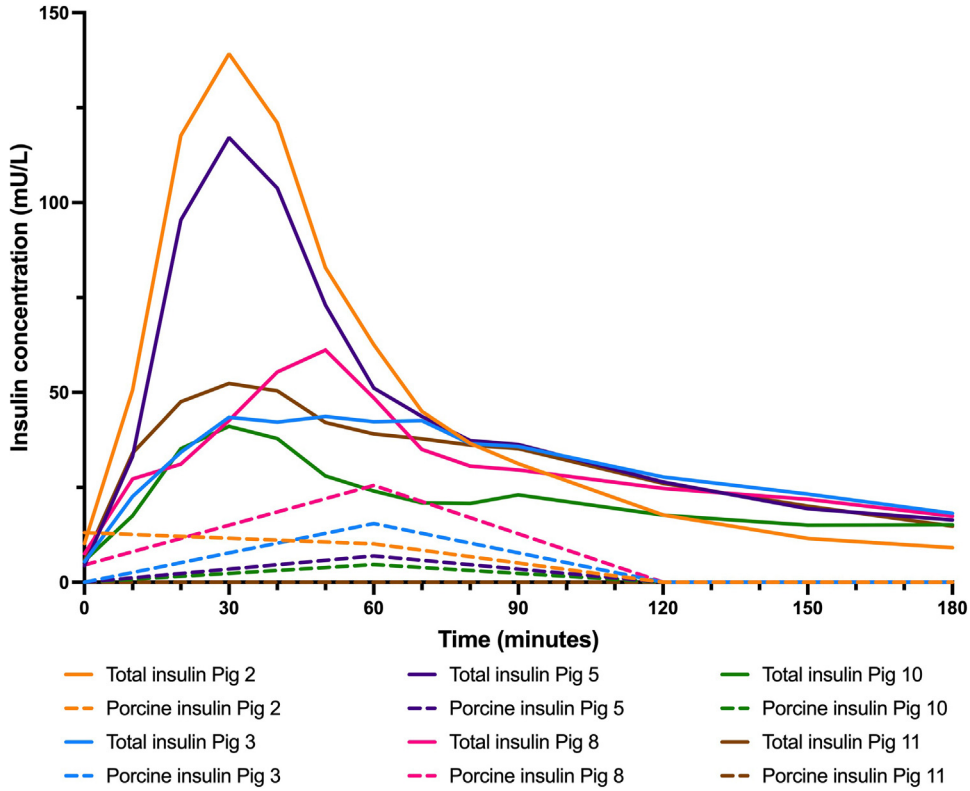

Fig 4. Total insulin concentrations in arterial plasma and porcine insulin concentrations in plasma in the glucagon group over time for all pigs.

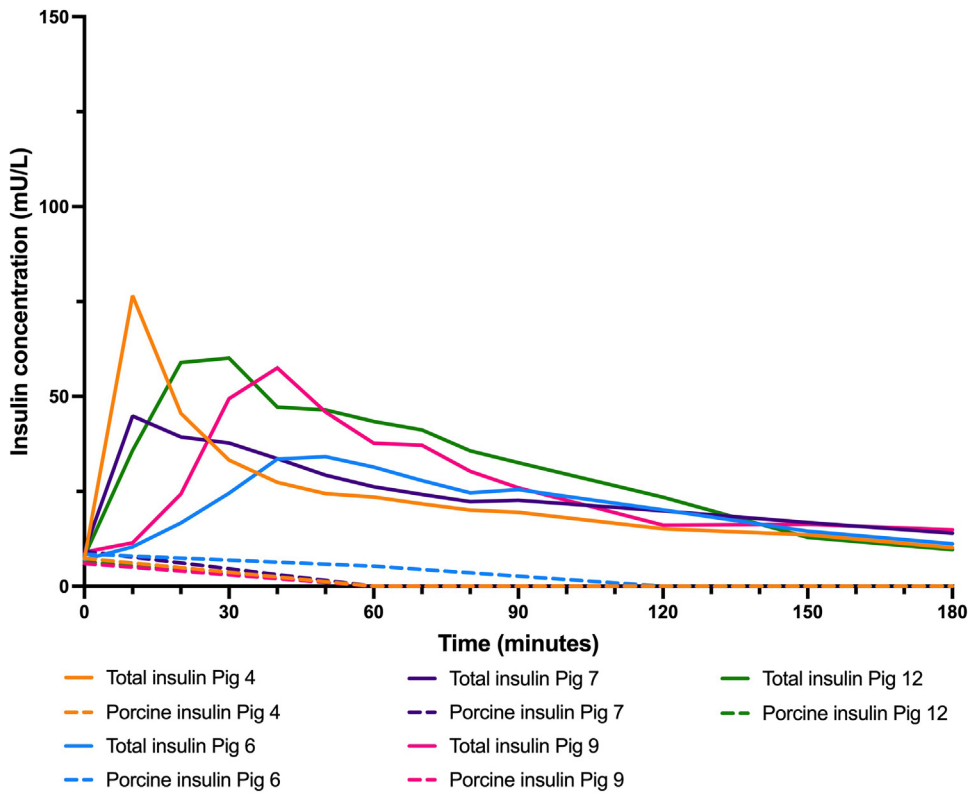

Fig 5. Total insulin concentrations in arterial plasma and porcine insulin concentrations in plasma in the placebo group over time for all pigs.

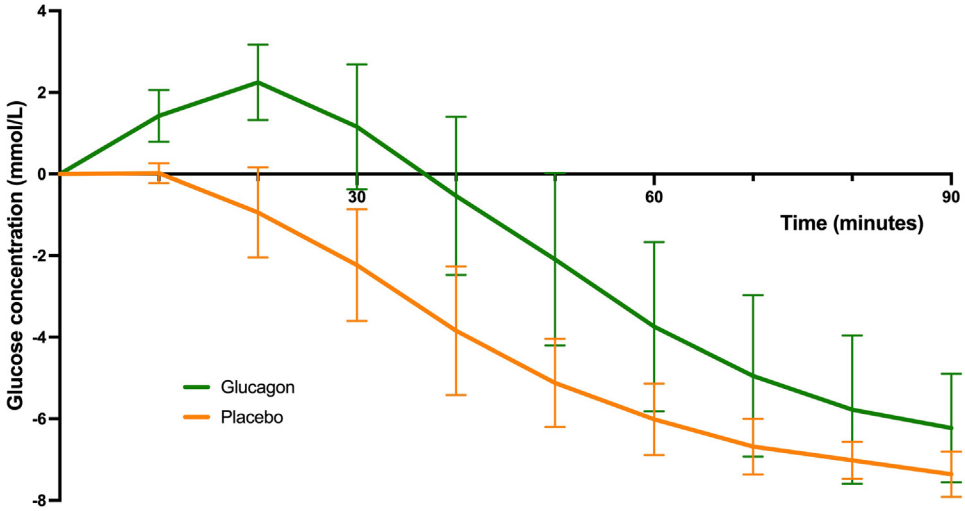

Fig 6. Change in mean arterial glucose concentration (with 95% CI error bars) over time in both study groups.

mean blood glucose concentrations than the placebo group at 10, 20, and 30 minutes after subcutaneous insulin administration ($P < 0.05$).

Discussion

This proof-of-concept study does not support our hypothesis that simultaneous glucagon and insulin administration shortens the absorption phase of insulin. However, the calculated total absorption of a single dose of insulin was significantly increased. Glucagon also caused a significant hyperglycemic effect the first 30 minutes after administration.

Subcutaneous absorption of insulin is positively correlated to local subcutaneous blood flow, and we believe that glucagon improves total insulin absorption through local vasodilation.[8,9] In a previous human trial, we found that injection of 10 and 100 µg glucagon caused a substantial increase in local subcutaneous blood flow after subcutaneous injection on the abdomen of healthy adults, evaluated with laser Doppler technology. For the 100 µg dose, median blood flow was increased by 250%,[5] whereas the 10 µg dose increased median blood flow by 145% (personal communication with M. K. Åm, by email to I. A. T. on October 11, 2022).

A logical objection to using glucagon to enhance insulin absorption is that glucagon antagonises the insulin effect on glucose metabolism and increases blood glucose concentrations. This is clearly demonstrated in the present study because blood glucose concentrations were significantly higher in the glucagon-treated group compared with the placebo group the first 30 minutes after insulin infusion.

Increasing postprandial hyperglycemia is highly undesirable and would contraindicate using glucagon to promote insulin absorption in a subcutaneous artificial pancreas. However, this could likely be avoided by using lower doses of glucagon with less potential to cause hyperglycemic effects. Studies into whether or not the local subcutaneous vasodilatory effects of glucagon are dose-dependent and at which threshold they occur should therefore be performed before investigating it as a method for accelerating insulin absorption in human beings.

There is cumulative evidence in the literature indicating that glucagon is released in relation to meals in patients with DM1. This effect is not present when an equal amount of glucose is given intravenously.[10–14] The secretion of glucagon increases within minutes after oral consumption of glucose or food. Glucagon from both the pancreas and the intestines is drained via the branches of the portal circulation to the liver. The liver is the main target organ for glucagon. It is therefore not surprising that there seems to be a considerable first-pass metabolism of glucagon in the liver; that is, that a large proportion of glucagon is extracted (and utilized) upon passing the liver and that only a small fraction reaches systemic circulation.[15] If this first-pass effect is saturable, as we have previously hypothesized,[16] microdoses of glucagon administered with mealtime insulin are probably less likely to cause postprandial glucose excursions because the liver will already be saturated with glucagon. This would have to be investigated in patients with DM1.

The values of T_{max} and $T_{1/2}$ of glucagon were comparable to observations in a previous pig study we have performed that investigated glucagon pharmacokinetics.[15] As such, the absorption and elimination time of glucagon did not seem to be influenced by insulin being deposited at the same subcutaneous site. No local adverse reactions on the skin were observed during the insulin and glucagon infusions. The volume of the saline and glucagon solutions that were injected in parallel with insulin in the present study was equal. Glucagon is stored as a compact, dry powder with lactose monohydrate, sodium hydroxide, and hydrochloric acid. It was dissolved in sterile water upon reconstitution. None of the excipients are known to interact with insulin aspart.

Strengths and limitations

A strength of the present study is that we used saline placebo injections. By this procedure we eliminated the possible volume effect at the site of subcutaneous insulin injection. The volume of the insulin injection was 0.1 mL and by adding 0.1 mL glucagon solution at the exact same site a dilution of the insulin solution may have influenced on the distribution of insulin aggregates between monomers and dimers that can be absorbed, and larger aggregates that cannot be absorbed. By using the same volume of saline as placebo, this possible volume effect on insulin aggregates, and thus on insulin absorption, was eliminated. However, the constituents of the 2 solutions differ, which might have influenced on the local subcutaneous degradation of insulin or the local subcutaneous distribution of insulin aggregates, and thereby also the insulin absorption.

A major limitation of this study is that the pigs were anesthetized, which disturbs many physiological processes and induces changes in circulation and metabolism.[17] Theoretically, anesthetic drugs and general anesthesia may also lead to alterations in the pharmacokinetics and pharmacodynamics of insulin. Experiments on awake pigs would have provided more reliable data. However, we considered it unethical not to anesthetize the pigs because the

experiments required precise subcutaneous drug administrations and frequent blood samplings. This would have exposed the pigs to unacceptable stress, fear, and pain if they were not anesthetized. Such stress and pain could have induced major physiological reactions, possibly of the same magnitude as anesthesia. We would like to point out that by any effect of anesthesia would likely be equal between the study groups.

Another important limitation is that we did not achieve full suppression of endogenous insulin secretion. We followed a protocol that provided adequate suppression in a previous pig study.[17] However, in the former study, we did not induce as high blood glucose concentrations as in the present, which likely stimulated endogenous insulin secretion. Endogenous insulin concentrations were slightly higher in the glucagon-treated pigs, probably because of the higher blood glucose concentrations in this group. However, the difference in $AUC_{0-\infty}$ of insulin remained significant also after subtracting $AUC_{0-\infty}$ of porcine insulin from the data. Still, it should be emphasized that the primary outcome of the study, faster insulin absorption, was negative and that larger $AUC_{0-\infty}$ was among several secondary outcomes of the study. The results should be interpreted cautiously because contribution from endogenous insulin cannot be ruled out.

This study had a very small sample size, with only 6 pigs in each study group. One pig in the placebo group had to be excluded from the analyses because of unintended intramuscular administration of insulin, which further reduced the power of the study. The small sample size opens the possibility that coincidence and outliers might significantly influence the results. More extensive and robustly designed studies into the vasoactive effects of glucagon and glucagon analogues and the potential for enhanced insulin absorption are warranted.

Conclusions

This proof-of-concept study does not support our previously published hypothesis that same-site subcutaneous administration of glucagon and insulin accelerates the absorption of insulin. However, we observed that glucagon increases the total amount of insulin absorbed after a single subcutaneous dose, which we believe is due to local glucagon-mediated vasodilation. This is a novel observation that provides the first indication that glucagon may act as a vasodilator and possibly enhance the absorption of subcutaneously injected insulin. If confirmed, this effect could be utilized in artificial pancreas devices. Further experiments are needed to identify the minimal effective dose of glucagon. Clinical studies in patients with DM1 should also be performed to study this promising new use of micro-doses of glucagon.

Declaration of competing interest

Norwegian University of Science and Technology, the university where the research was conducted and where the researchers reside, has a patent filed related to the research. S. Carlsen and S. Christiansen are among the inventors. The authors have indicated that there are no other conflicts of interest regarding the content of this article.

Acknowledgments

All authors contributed to the conception of the study. IAT and MKÅ was responsible for the conduction of animal experiments and data collection. IAT wrote the protocol, analysed, and interpreted the data and wrote the first draft of the manuscript. All authors reviewed and commented on previous versions of the manuscript. All authors have read and approved the final version of the manuscript.

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