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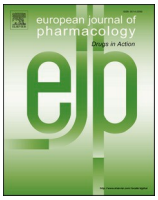

The effect of glucagon on local subcutaneous blood flow in non-diabetic volunteers; a proof-of-concept study

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ABSTRACT

Introduction: Glucagon and insulin are the two most important hormones in glucose metabolism and have been incorporated in the dual-hormonal artificial pancreas, a device for automated glucose regulation for people with diabetes type 1. Currently the subcutis is the preferred site of hormone delivery for insulin-only as well as dual-hormonal artificial pancreas systems. The delay in glucose-lowering effect after subcutaneous injection of insulin is substantial, in contrast to the elevation of blood glucose values after subcutaneously injected glucagon which is occurs shortly after injection. We hypothesize that this is caused by properties of glucagon and have investigated the vasodilative effect of glucagon on subcutaneous blood flow in this proof-of-concept study.
Methods: Twenty-two volunteers received subcutaneous injections of 0.1 mg and 0.01 mg glucagon, and saline on the abdomen. Blood flow was measured by a laser doppler blood flowmeter for 35 min after injections.
Results: Injection of 0.1 mg glucagon resulted in a significant increase in blood flow compared with baseline blood flow for all time intervals. Significant increase was also observed after the 0.01 mg glucagon injection, except between two- and five-min post injection. The inter-individual variance was large and a third of the subjects did not show an apparent increase in local subcutaneous blood flow after the 0.1 mg glucagon injection.
Conclusion: This proof-of-concept study shows that micro-boluses of glucagon increases local subcutaneous blood flow on the abdomen of non-diabetic subjects. However, the vasodilative effect of glucagon is not observed in all subjects. The trial was not registered to protect intellectual property rights.

1. Introduction

Glucagon plays an important role in glucose metabolism and is released to elevate blood glucose levels in times of imminent hypoglycaemia. The hormone is traditionally used as a drug to correct severe hypoglycaemia. In recent years glucagon has also been incorporated in systems for automated insulin delivery, i.e., artificial pancreas (AP), to automatically regulate the blood glucose (BG) levels of people with diabetes mellitus type 1 (DM1). An AP should ease and adjust insulin delivery, improve overall blood glucose (BG) control, and thus improve the quality of life for DM1 patients. Studies have shown some advantages with AP systems in terms of more time spent in the therapeutic range and less time spent in hyper- and hypoglycaemia (Bekiari et al., 2018; Karageorgiou et al., 2019). However, modern APs have time delays in insulin-effect and glucose-feedback which prevents adequate performance, and the systems are not yet fully automated nor able to achieve optimal glucose control.

The delay in subcutaneous (SC) insulin absorption and effect on BG levels is the major contributing factor to why a fully automatic AP is not yet developed. Even with the most fast-acting meal insulins there is a delay of 90 min before the major BG lowering effect is observed (Evans et al., 2019). The delay after SC injection of glucagon is not so pronounced. The maximum glucose elevating effect is observed after 60 min (Blauw et al., 2016; Hövelmann et al., 2019). Our research group, Artificial Pancreas Trondheim, have performed animal experiments to investigate the pharmacokinetics and pharmacodynamics of IP glucagon delivery. In our studies, we found the speed of absorption of glucagon to be similar between SC and IP delivery (Dirnena-Fusini et al., 2018; Åm et al., 2020), contrary to the major delay seen after SC delivery of insulin (Christiansen et al., 2017). Glucagon is known to relax smooth muscle

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cells in the airways and gastrointestinal tract (Pollack, 1993). Thus, we hypothesised that the similar SC compared to IP absorption of glucagon could be facilitated by the same effect on vascular smooth muscle cells at the site of glucagon injection in the SC tissue, resulting in a vasodilative effect that enhances SC absorption of glucagon itself. Although only a hypothesis at present, if it proves correct, and as glucagon already would be a part of a dual hormone AP system, utilizing its vasodilative properties would not require any add-ons to the AP system but would potentially minimise the delay in insulin effect, thus enabling a fully automated AP with superior glucose control.

To date, no study has specifically investigated the use of micro-doses of glucagon as a vasodilative drug to increase SC blood flow. Therefore, our proof-of-concept study aimed to investigate the possible vasodilative properties of glucagon on SC perfusion in non-diabetic subjects.

2. Materials and methods

2.1. Study design

We performed a single-blinded, randomized, placebo-controlled study with cross-over design on 22 volunteers. Each subject participated for one session of approximately 2 h where they received two SC injections of glucagon and one SC injection of saline. Local cutaneous blood flow was measured with a laser doppler blood flowmeter. The primary outcome was the subsequent change in local blood flow.

2.2. Study subjects

The study subjects were between 18 and 65 years of age and reported no known disease for the last three months. Exclusion criteria were defined as known major chronic illness, chronic skin disease, pregnancy, and subjects regarded not eligible to participate for any reason by the responsible physician.

2.3. Study procedures

Participants were discouraged from conducting activities that would potentially affect the blood flow in the skin prior to the examination. This included physical activities, smoking, and high consumption of caffeine. The room temperature was around 24 centigrade and the participants were allowed to wear a blanket if they felt cold during the experiments. Information on blood pressure, age, height, and weight was collected from each participant at the start of the examination. Blood pressure was also measured at the end of the procedure. Participants were positioned in a supine position on a bench and the probe of the laser doppler attached to the skin of the abdomen with self-adhesive tape. Injection sites were localized approximately 10 cm lateral to the umbilicus on one side, and 2.5 cm up and 2.5 cm down from the corresponding point on the other side of the abdomen. Glucagon (Novo Nordisk, Bagsværd, Denmark) was mixed with 0.9% saline to concentrations of 1 mg/ml and 0.1 mg/ml at the start of the day. Saline (0.9%) was used as placebo. The injections were performed with insulin syringes (BD Micro-FineTM) with a 30G needle with a length of 8 mm. The skin was perforated 2–3 mm from the laser probe, and the needle was inserted at approximately 45-degree angle to get as close to the probe centre as possible. The volume of all interventions was 0.1 ml, and all injections were performed over 10–15 s.

The three interventions were performed consecutively on altering sides of the abdomen. The order of interventions for each subject was randomized using a virtual dice (https://no.piliapp.com).

Baseline measurements were sampled for 4 min before injection, and the average blood perfusion unit (BPU) between 30 s and 210 s was used as the baseline value for each specific measurement. Post injection values were measured for 35 min and directly compared to the average baseline value measured before injection.

2.4. Laser Doppler flowmetry

Cutaneous blood flow was measured using a blood flowmeter and a miniature surface probe with a diameter of 12 mm (AD Instrument, Oxford, United Kingdom). These instruments are designed and approved for non-invasive measurements of cutaneous blood flow in clinical trials. The probe illuminates the skin with visible light (830 nm $\pm$ 10 nm, effect $<$0.5 mW) through an optic fibre. Light is reflected by moving and static structures and registered through another optic fibre in the same probe. The output signal from this instrument is BPU, an arbitrary unit representing the perfusion of blood cells in the illuminated area by utilizing the doppler shift in the reflected light caused by cells in motion. The method cannot distinguish between increased velocity of the blood cells and increased number of moving cells.

Data was recorded and stored in the software program LabChart8 (AD Instruments, Oxford, United Kingdom). The sampling rate was set to 10 BPU measurements per second and data were continuously sampled throughout the procedure.

Data were analysed using MATLAB R2020b. The time series after injection was divided into time intervals of 1-min intervals from 0 to 5 min and 5-min intervals for the rest of the post intervention registration. BPU values after interventions are expressed as percentage change in BPU compared to baseline.

2.5. Statistical analysis

All statistical analyses were performed in MATLAB R2020B. Sample size was not calculated because of the proof-of-concept design. Normality of data was tested using Anderson Darling test (adtest). As the data did not follow any specific distribution, a right-tailed Wilcoxon signed rank test was used to analyse the differences between the 3 interventions for each time interval individually, identifying time intervals where one intervention had significantly greater BPU-responses than the other. We compared injections of glucagon 0.1 mg to saline and glucagon 0.01 mg to saline. P-values $<$ 0.05 were considered statistically significant. A right tailed Wilcoxon rank sum test was used to compare responder and non-responder groups, sex groups, and BMI groups. No adjustment for multiple testing was performed.

Results are presented as medians with first and third quartiles (Q_1 and Q_3), if not otherwise specified.

2.6. Ethical considerations

The study was approved by the Regional Ethical Committee (REC) with approval number #180201. All participants provided written informed consent before participating. A physician was available by cell phone during all experiments.

3. Results

During April and May 2021, 22 volunteers participated in the study. No participant was excluded based on the pulse- and blood pressure measurements before intervention. There were no reports of adverse effects of glucagon injection or the procedure in general. The baseline characteristics of study participants are presented in Table 1.

Table 1
Baseline characteristics of study participants.

Characteristics	Mean (range)
N (male/female)	22 (8/14)
Age (years)	32 (22–63)
BMI (kg/m^2)	26.1 (19.3–35.4)
Blood pressure before intervention (mm Hg)	134/84 (107/63–176/118)
Blood pressure after intervention (mm Hg)	129/81 (107/65–163/122)

Continuous data are given as mean (range).

3.1. Injection of 0.1 mg glucagon, 0.01 mg glucagon, and 0.9% saline

All interventions resulted in temporarily elevated BPU values, where the 0.1 mg glucagon injection resulted in the highest response, 0.01 mg to a lesser degree and the saline injection gave the weakest response (Fig. 1). The pure effect of glucagon is showed in Fig. 2, where the effect of the saline injection is subtracted from the results of the 0.1 mg and 0.01 mg glucagon injections.

Maximal BPU effect was observed 4, 2 and 2 min after the injection of 0.1 mg glucagon, 0.01 mg glucagon and saline, respectively.

There are significant differences in post injection BPU values for all time intervals comparing the 0.1 mg glucagon and saline injections ($p < 0.005$ for all intervals), and for eight out of 11 time intervals when comparing the 0.01 mg glucagon and saline injections ($p < 0.01$ for intervals 0–1 min, 1–2 min, 15–20 min, 20–25 min, and 25–30 min and $p < 0.03$ for intervals 5–10 min and 10–15 min). Intervals between 2 and 5 min were not significantly different compared with saline injection.

The median (Q_1-Q_3) baseline BPU values were 37 (33–45) BPU, 36 (33–44) BPU and 37 (31–43) BPU before the injection of saline, 0.1 mg glucagon and 0.01 mg glucagon, respectively.

3.2. Responders vs. non-responders

When the results were analysed, it became apparent that some subjects showed no greater response to the 0.1 mg glucagon injection than to the saline injection. Consequently, we performed a post hoc classification dividing the subjects into responders and non-responders based on their response to the 0.1 mg glucagon injection with the effect of saline removed. The criteria for classification as a responder was the fulfilment of the following:

1. 6/6 positive BPU values in the first 6 time-intervals (i.e., higher BPU values than saline)
2. For at least 2 time intervals, the effect of glucagon 0.1 mg minus the effect of saline being >100% of baseline BPU values.

Fifteen subjects were defined as responders and seven subjects were defined as non-responders. The BPU responses for the two groups are shown in Fig. 3.

While the BPU values were significantly higher in the responder group compared to the non-responder group for injection of 0.1 mg glucagon ($p < 0.01$ for all time intervals), there were no significant differences in BPU response for the 0.01 mg glucagon dose or the saline injection for any time interval.

The median (Q1-Q3) baseline BPU values before interventions were 36 (32–46) BPU and 37 (34–42) BPU in the responder and non-responder groups, respectively.

3.3. BMI and sex

There were no statistically significant differences for any of the time intervals for the different interventions comparing females and males or comparing subjects with a BMI$\leq$ 25 kg/m^2 to those with BMI >25 kg/m^2 (Table 2).

4. Discussion

In this proof-of-concept study, we observed that small doses of glucagon injected SC on the abdomen of non-diabetic subjects increased the local SC blood flow. Injection of 0.1 mg glucagon resulted in the highest BPU response with a maximum median increase of 250%. Injection of 0.01 mg glucagon also increased the local SC blood flow, although to a lesser degree. Our observations are comparable with observations made by Simmons et al. who reported 300–500% increase in SC blood flow after SC injection of 1 mg glucagon (Simmons and Williams, 1992). However, while Simmons et al. reported the maximum effect 20 min after injection of a ten to hundred times larger bolus of glucagon, we observed the maximum blood flow response already 2–4 min after a micro-bolus of glucagon. Another contrasting finding in our study was the declining effect on local blood flow. Simmons et al. showed the blood flow effect to remain high throughout the 30-min-long

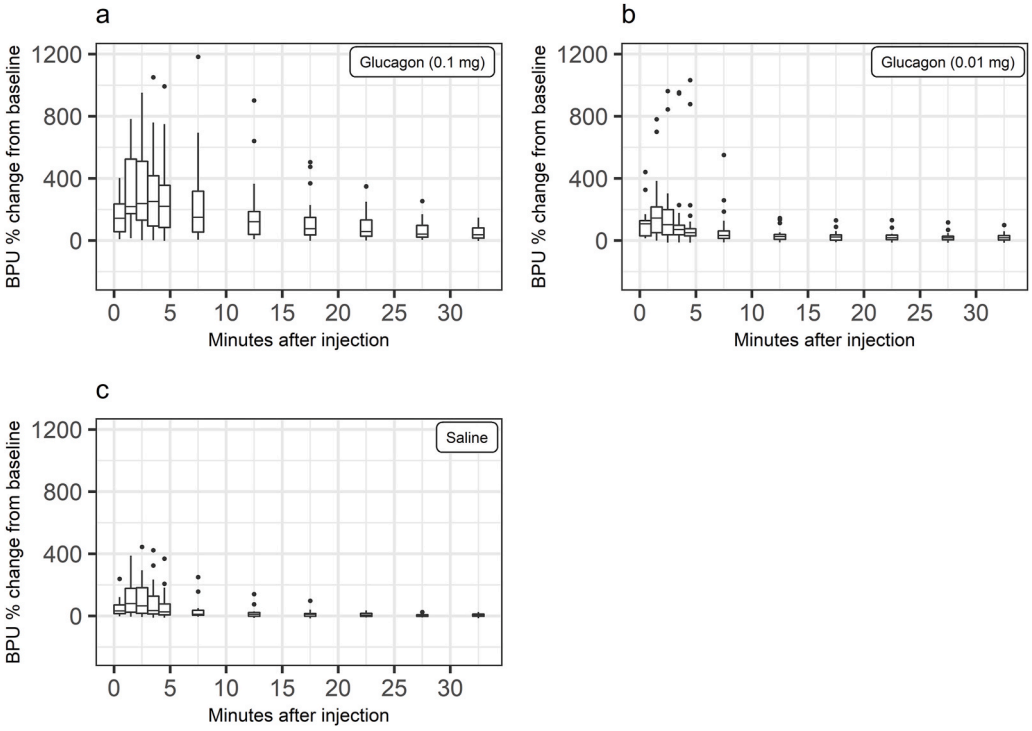

Fig. 1. Boxplot of BPU values given as % change from baseline post injection of 0.1 mg glucagon (a), 0.01 mg glucagon (b) and 0.9% NaCl (c) in 22 volunteers. Time intervals of 1-min was used for the first 5 min, thereafter 5-min intervals were used. Whiskers represents values located less than 1.5 times the interquartile range (IQR) from the median. Datapoints located more than 1.5 times the IQR from the median are presented as outliers.

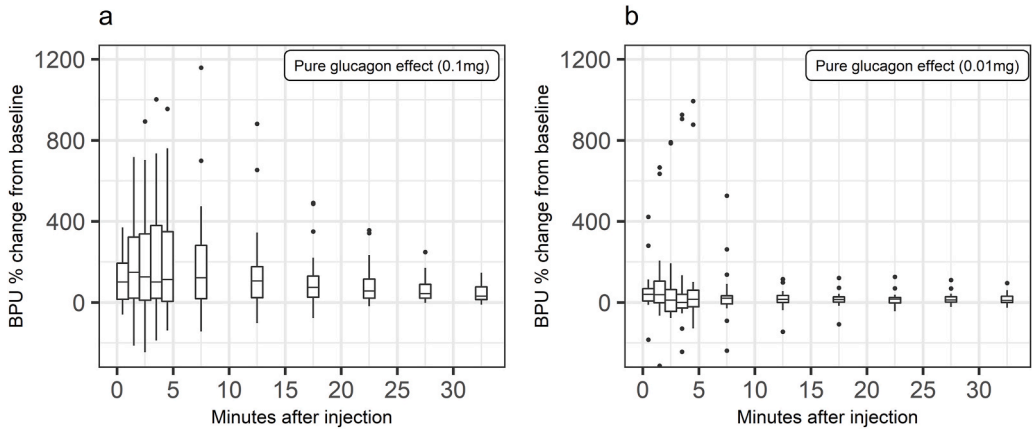

Fig. 2. Boxplot of BPU values given as % change from baseline post injection of 0.01 mg glucagon (a), and 0.01 mg glucagon with the effect of 0.9% saline injection subtracted in 22 volunteers. Time intervals of 1-min was used for the first 5 min, thereafter 5-min intervals were used. Whiskers represents values located less than 1.5 times the interquartile range (IQR) from the median. Datapoints located more than 1.5 times the IQR from the median are presented as outliers.

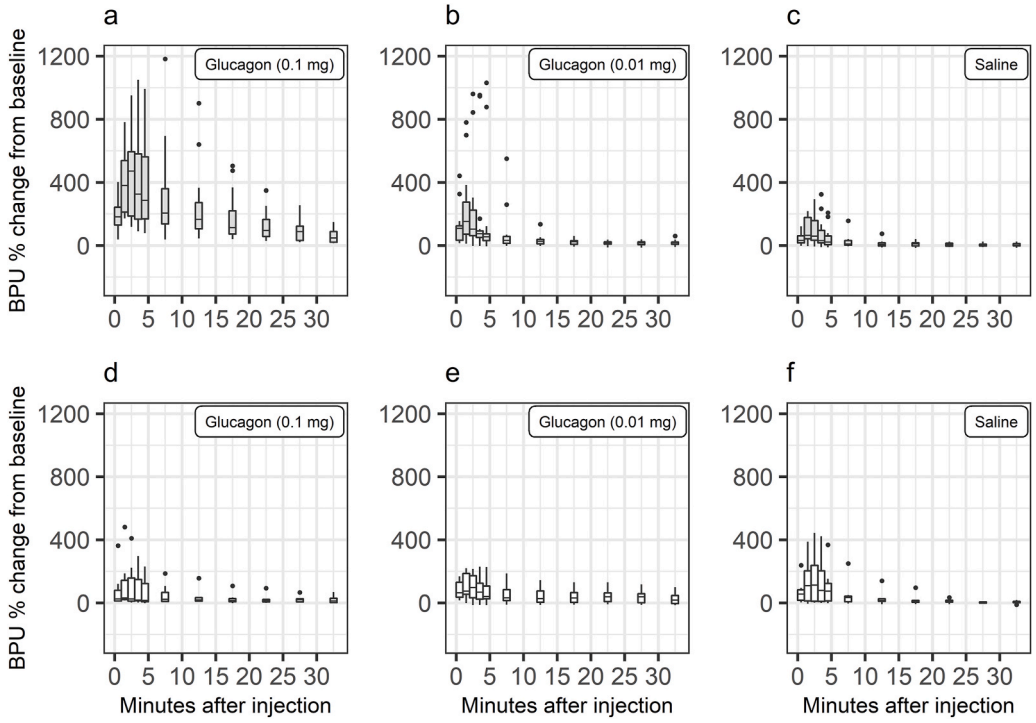

Fig. 3. Boxplot of BPU values given as % change from baseline post injection for responders on the top row (n = 15) and non-responders on the bottom row (n = 7). Time intervals of 1-min was used for the first 5 min, thereafter 5-min intervals were used. Whiskers represents values located less than 1.5 times the IQR from the median. Data points located more than 1.5 times the IQR from the median are presented as outliers.

Table 2
Maximum BPU response according to sex and BMI.

Maximum BPU achieved - median (Q_1-Q_3)			
	Glucagon 0.1 mg	Glucagon 0.01 mg	Saline
Females	342 (183–534) %	144 (66–213) %	79 (41–151) %
Males	194 (29–299) %	141 (41–214) %	112 (12–187) %
BMI ≤25	451 (237–555) %	144 (96–255) %	146 (29–199) %
BMI >25	201 (118–361) %	130 (41–204) %	60 (26–150) %

Maximum BPU response given as % change from baseline after injection of glucagon 0.1 mg, glucagon 0.01 mg and saline in female subjects (n = 14), male subjects (n = 8), subjects with BMI ≤25 kg/m^2 (n = 8), and subjects with BMI >25 kg/m^2 (n = 14). Results are presented as medians with quartile spans.

experiment. In our study, however, the BPU values slowly declined after the initial peak in blood flow. Even so, the blood flow was still increased 30–35 min after the 0.1 mg glucagon injection with a median increase in BPU of 31% when the effect of saline injection was subtracted.

Perforating the skin with a needle and injecting a volume causes mechanical trauma and triggers several biological responses, including the release of nitric oxide and histamine, both of which are potent vasodilators (Præstmark et al., 2014), and these effects are apparent in our results. However, the increase in BPU after the saline injection was significantly smaller compared with the injection of 0.1 mg glucagon, and for most of the time intervals after the injection of 0.01 mg glucagon. As each participant acted as their own control, we can report the pure vasodilative effect of glucagon by removing the effect of the saline injection.

Glucagon is mainly known for its glucose elevating properties. However, the hormone also affects smooth muscle cells and is used to relax the gastrointestinal tract before imaging procedures, such as abdominal magnetic resonance imaging, and to relax the oesophagus in case of oesophageal food impactions (Bazot et al., 2021; Lake et al., 2021). Another known effect of glucagon is its ability to reduce vascular resistance in several organs. Glucagon decreases vascular resistance in splanchnic and hepatic vessels, and large doses of glucagon can increase renal blood flow, glomerular filtration rate and renal excretion (Farah, 1983; Nakahara et al., 1997). However, the vasodilative effect seems to differ according to site, as no vasodilative effect was observed in the femoral artery (Nakahara et al., 1997). Animal studies have also shown that glucagon dilates renal, mesenteric, and coronary arteries, as well as the thoracic aorta, and potentiates reperfusion of coronary arteries after an ischemic event (D'Orléans-Juste et al., 1985; Moir and Naylor, 1970; Okamura et al., 1986; Rosic et al., 2010; Sélley et al., 2016). The precise mechanisms of glucagon's vasodilative properties are not known. However, it is proposed to be a direct, endothelium-independent effect on vascular smooth muscle cells, causing an increased production of cyclic adenosine monophosphate (cAMP) (Okamura et al., 1986; Sélley et al., 2016). Another suggestion is that glucagon indirectly affects the release of histamine and nitric oxide (Sélley et al., 2016). As of today, available literature does not provide conclusive results.

Although the mechanism of effect is not completely known, our proof-of-concept study clearly indicates that glucagon increases the local blood flow just beneath the skin surface. Cutaneous blood flow can go from almost zero to 6–8 L/min during thermal stress (Charkoudian, 2003), and plays an essential role in thermoregulation. Various internal and external thermal stimuli are the main regulatory factors for temperature control. However, the regulatory system of cutaneous blood flow is complex and numerous known and unknown factors also affect the cutaneous blood perfusion, such as age, sex, smoking, and various diseases. The vessels in the skin are organised in three well-connected horizontal plexuses (Raju et al., 2012). The most superficial network is located 1–1.5 mm below the skin surface and supplies the dermis with blood via the dermal papillary loops. The superficial plexus is divided into vascular units comprised of one ascending arteriole from the middle plexus and a paired descending venule, with some lateral communication between vascular units. The lower vascular network is located within the SC tissue and supplies the upper two networks.

The ascending arterioles connecting the two most superficial horizontal plexuses are randomly placed with 1.5–7 mm distance (Braverman et al., 1992), and the capillaries have been shown to be more sparsely located on the abdomen than in other anatomical areas (Raju et al., 2012) especially in obese subjects (Altintas et al., 2016). The possibility of accidently avoiding these vessels when injecting glucagon and measuring the BPU, may have contributed to the observed inter- and intra-individual variations. The distribution of SC blood vessels might also differ among individuals. However, we cannot exclude the existence of major individual differences in the vasodilative response to glucagon, which would explain the observed inter-individual variation in BPU response.

Interestingly, while not statistically significant, the responder group showed a greater initial BPU increase also to the injection of 0.01 mg of glucagon compared with the non-responder group. In contrast, the response to saline was almost equal between the two groups. The fact that the two groups reacted differently to glucagon but similarly to saline, suggests that the hyperaemic glucagon effect observed in the responder group is independent of the trauma- and volume related effects, and indicates that the mechanism of SC vasodilation differs for glucagon and saline.

Enhancing insulin absorption by increasing local SC blood flow is not a new idea, and Lilly recently introduced a new formulation of insulin containing treprostinil, a powerful vasodilative drug, to speed up the absorption of insulin. Taking advantage of the vasodilative properties of glucagon can potentially increase insulin absorption without adding complexity to the dual hormone AP system. However, a possible challenge with this use of glucagon is its glucose elevating effect. The effect on BG levels was not investigated in this study. However, smaller doses of glucagon have been studied to treat milder forms of hypoglycemia, and one study reported that a dose of 0.1 mg glucagon injected SC corrected mild hypoglycemia by increasing the blood glucose by 2.1 mmol/L without resulting in rebound hyperglycemia (Ranjan et al., 2016). An in-silico study proposes the optimal glucagon dose to treat mild hypoglycemia to be exponentially related to serum insulin concentrations, where higher insulin levels required higher glucagon levels to increase plasma glucose (Ranjan et al., 2018). Clinical studies have also found that glucagon's ability to elevate blood glucose levels are blunted and sometimes inhibited by high concentrations of insulin (El Youssef et al., 2014, Bakhtiani et al., 2015). This suggests that if glucagon is used to enhance the absorption of insulin, a low dose of glucagon will have less impact on the blood glucose compared to situations when glucagon is given when insulin levels are low. However, but the effect of BG levels must be investigated in patients with DM1 before any conclusions of this intervention can be decided.

This proof-of-concept study was not prospectively registered in any ICMJE approved clinical trial registry to protect the intellectual property of the idea while applying for a patent. The study included only 22 participants and only two injections of glucagon per subject, giving a rather limited sample size. However, making each participant his/her own control and randomizing the injections are strengths of the current study. This is supported by our observations that the participants responded differently to glucagon, while responding similarly to saline, leading to the classification of responders and non-responders.

Another limitation of the study is the small spatial area of the skin, which is illuminated by the LDF, and the uncertainty of both the depth of the blood flow measurements and injections. With our set up, the sampling depth is likely in the range of 1–1.5 mm (ADInstruments, 2021), while glucagon and saline was injected approximately 5.5 mm beneath the skin surface. This is far below the depth that LDF measures blood flow. Nevertheless, the vascular network of the SC tissue supplies the dermal vessels (Raju et al., 2012), and it is known that skin surface LDF-measurements correlate well with ^{133}Xe-washout methods for estimation of blood flow in SC tissue (Wellhöner et al., 2006), the gold standard for measuring SC blood flow (Frayn, Karpe, 2014). For individuals with low BMI, there is a chance that the glucagon was deposited in the facia rather than in the SC tissue. We did not measure the thickness of the SC tissue; but inserting the needle with an angle of 45° results in a deposition depth of 5.5 mm, which correlates most likely to the lower part of the SC tissue (Gibney et al., 2010).

The aim of this study was to investigate whether small SC doses of glucagon increases the local blood flow in non-diabetic subjects. This is the first study, which specifically investigates the SC vasodilative properties of small doses of glucagon. Consequently, there are many questions that need to be investigated. As we propose to utilize the vasodilative effect of glucagon to improve the treatment of DM1, this effect must be investigated in subjects with DM1. It is also important to study people with various duration of DM1, as prolonged diabetes influence on the microvasculature and could potentially affect the vasodilative effect of glucagon.

5. Conclusion

In non-diabetic subjects, SC injected low doses of glucagon significantly increases local SC blood flow. However, it might seem that one third of subjects are non-responders. Further investigations as well as larger and more sophisticated trials are needed to investigate whether the effect is transferrable to people with DM1 and thus be able to influence the ultimate primary outcome, improved SC insulin absorption. In such trials, glucagon analogues and new formulations of glucagon should also be tested.

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Authorship

All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

Compliance with ethics guidelines

The study was approved by the Regional Ethical Committee (REC) with approval number #180201, and was performed in accordance with the Helsinki Declaration of 1964, and its later amendments. All participants provided informed consent to participate in the study.

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Marte Kierulf Åm: Methodology, Visualization, Writing – original draft, Writing – review & editing. **Erlend Yttersian Munkerud:** Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing. **Mathilde Hallem Berge:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Sverre Christian Christiansen:** Methodology, Resources, Writing – review & editing, Funding acquisition. **Sven Magnus Carlsen:** Conceptualization, Supervision, Project administration, Methodology, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors SMC and SCC are involved in a patent application on the use of micro-dosed glucagon to enhance the absorption of insulin and performance of subcutaneous continuous glucose sensing.

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