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Research article

Towards a Göttingen minipig model of adult onset growth hormone deficiency: evaluation of stereotactic electrocoagulation method

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ABSTRACT

Background: Adult onset growth hormone (GH) deficiency (AGDH) is a potentially underdiagnosed condition, caused by damage to the pituitary gland. AGHD is treated with growth hormone replacement therapy. A large variety of clinical symptoms and changes in the metabolic homeostasis can be observed and quantified. New large animal models are needed for future drug development.

New method: In this study, we evaluate methods for a new large non-primate animal model of GH deficiency in post pubertal Göttingen Minipigs (minipig). Lesions in the pituitary gland were made by stereotaxic monopolar thermo-coagulation guided by magnetic resonance imaging (MRI), and pituitary function was evaluated using insulin tolerance test (ITT) with measurements of growth hormone secretion induced by hypoglycemia.

Results: Lesions were successfully applied to the pituitary gland without any damage to surrounding tissue including the hypothalamus, which was confirmed by post-operative MRI and post mortem histology. Plasma levels of GH during ITT showed no decrease in secreted levels one week after surgery compared to levels obtained before surgery.

Comparison with existing methods: Compared to other GH insufficiency models, eloquent brain tissue is spared. Furthermore, alternatively to rodent models, a large animal model would allow the use of human intended equipment to evaluate disease. Using the minipig avoids social, economical and ethical issues, compared with primates.

Conclusion: The lesions did not remove all GH production, but proof of concept is demonstrated. In addition, the ITT is presented as a safe and efficient method to diagnose GH deficiency in minipigs.

1. Introduction

Traumatic head injuries, tumours, neurosurgical operations or other pathological conditions in the pituitary gland may lead to AGHD. GH levels are often the first to be affected by such conditions. AGHD is successfully treated with GH replacement therapy (Fukuda et al., 2014; Stochholm et al., 2006), however, AGHD is probably underdiagnosed and thus untreated (Karaca et al., 2016) as the manifestations are clinically subtle, develops over a long time and may be similar to other disorders such as the metabolic syndrome (Møller and Jørgensen, 2009). AGHD is associated with reduced bone mineral content and density, increased waist circumference, increased body mass index (BMI), altered metabolism, dyslipidemia, premature atherosclerosis, increased fat mass, lower lean body mass, insulin resistance, increased prevalence of metabolic syndrome, increased mortality, impaired cognitive function and reduced quality of life (Appelman-Dijkstra et al., 2013; Ayuk and

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Sheppard, 2006; Brod et al., 2014; Claessen et al., 2014; Fukuda et al., 2014; Johansson et al., 1995; Kreitschmann-Andermahr et al., 2010; Rosén et al., 1993; Stochholm et al., 2007; Svensson and Bengtsson, 2009; Uzunova et al., 2015). The majority of symptoms can be alleviated or normalized by GH replacement therapy, however, long-term surveillance of side effects and individualized dose regulation is required (Drake et al., 1998; Johannsson et al., 1997; Nilsson et al., 2007; Toogood, 2005).

Currently, however, there is no well-established adult onset non-rodent model (Appelman-Dijkstra et al., 2014; Fukuda et al., 2014; Gahete et al., 2016; Höybye and Christiansen, 2015; Li et al., 2015; Miller et al., 2010).

GH analogues should be tested in a non-rodent animal in addition to a rodent model in order for the response to be regarded as consistent in different species, including humans. Thus, a non-rodent, large animal model with pituitary hormone deficiency that can be subjected to long-term replacement therapy will be of great value in the GH research field (Appelman-Dijkstra et al., 2014; Hansen et al., 2002; Thorsted et al., 2016). Surgical hypophysectomy has long been the standard for inducing pituitary hormone deficiencies in animal models and has primarily been performed on immature pigs, rats and mice for short term monitoring regimens. Using a temporal surgical approach, animals could not eat after surgery, probably due to jaw-muscle and jaw-joint pain (Amet et al., 2010; Kwak et al., 2009; Link and St Clair, 1954; Zhang et al., 2012).

The aim of this study was to test a new surgical method for inducing AGHD in post pubertal minipig, evaluate the insulin tolerance test as diagnostic tool in the minipigs and evaluate hormone replacement treatment for pituitary hormones besides GH.

It is however important to remember that the model is translational, so differences on hormonal receptors is needed to make human intended drug testing, and further receptor analysis is important (Hinrichs et al., 2018).

2. Methods

2.1. Choice of animal model

The Danish Animal Expectorate ("Dyreforsøgs tilsynet") ethically approved the experiments.

The minipig is a relevant model in GH deficiency research due to the anatomical, physiological and to some extent hormonal (Louveau and Gondret, 2004) resemblance to humans and the well characterized anatomical and physiological similarities (Lind et al., 2007). In humans, hypophysectomy by a transsphenoidal approach is performed routinely, but access by this route is hindered by the snout and tight jaw of the pig (Pinar et al., 2015; Solari et al., 2014). Four minipigs were included in the present study. To ease the procedures and reduce stress in the pigs, they were accustomed to interactions with humans by petting and time spent in their pen with researchers. Animals were housed in adjacent pens with snout contact to neighbouring animals. The minipigs were fed (special Diets Services-pellets(SDS)) strictly based on animal weight and had water *ad libitum*. Prior to surgery, the animals were fasted for 16 h. They were housed at 20 °C air ventilation with 50–55% humidity, and with a day/night cycle of 12 h.

2.2. Study design

Catheters were inserted one week prior to the pituitary surgery and ITTs were performed 3 days before and 7 days after pituitary surgery. The minipigs received hormone replacement therapy in the week from the pituitary surgery to the final ITT. Analgesics were given for 3 days after the catheter insertion and for 4 days after the pituitary surgery as illustrated in Fig. 1. The minipigs were euthanized after the last ITT by anaesthesia with 0,6 ml/kg Zoletil mixture and a subsequent pentobarbital overdose in the catheter.

2.3. Surgical procedure

A magnetic resonance imaging (MRI) guided stereotactic electrocoagulation approach was chosen based on previous research (Bjarkam et al., 2009, 2004; Glud et al., 2017) (Fig. 2). The minipigs were sedated

Fig. 2. The experimental setup for hypophysectomy with the minipig placed in the stereotaxic localizer box. The active electrode (**) and return electrode (*) are marked.
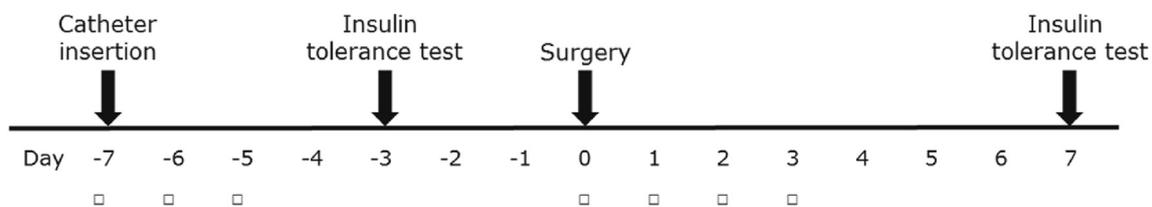
□: *Analgesic: Catheter insertion: oral meloxicam (0,02 ml/kg, 24 hours coverage) once daily for 3 days. Surgery: buprenorphine (0.1 mg/kg, 8 hours coverage) before and after the procedure and oral meloxicam (0.02 ml/kg, 24 hours coverage) once daily for 4 days.*

●: *Hormone replacement therapy: 25 mg Cortisol and 3 ug desmopressin daily as well as 0.25 mg hydro-thyroxine twice weekly*

Fig. 1. Study design. Open square: analgesic, Black dots: hormone replacement therapy.

with 10 ml of Midazolam (B. Braun, 5 mg/ml) SC before transportation from the animal facility to the surgical facility. Prior to oro-pharyngeal intubation (using a tube size 4–6 depending on weight and anatomy) anaesthesia was initiated using 5–10 ml IV of 70% Midazolam (B. Braun, 5 mg/ml) and 30% S-Ketamine (Pfizer, 25 mg/ml). Furthermore 5 ml Cefuroxim IV (150 mg/ml) was used as prophylactic preoperative antibiotic, and 1 ml buprenorphine (Temgesic®) (0.3 mg/ml) IM was administered as pain relief before the procedure (Ettrup et al., 2011).

The minipigs were ventilated manually until stabile respiration was established, and then automatically ventilated with a mixture of air and O_2 (60%/40%) and 0.5–2.5% Sevofluran (Baxter) to maintain the general anaesthesia. During transportation in the facility the anaesthesia was maintained using propofol (Propofol "B. Braun") infusion (2–5 mg/kg/min). The sedated and intubated minipig was placed and fixed in a stereotactic localizer box (NeuroLogic Mark2, Denmark).

Local analgesia of the Zygoma region (the temple) was obtained by infiltration of 2.5 ml 5% Lidocaine (SAD) prior to fastening of the MRI-compatible localizer box with titanium screws. A fiducial marker was placed as previously described (Glud et al., 2017). The minipig were MRI scanned next door to the operation facility, and brain MRI's were 3D-reconstructioned in order to localize the pituitary gland using stereotaxic surgery planning software (Surgiplan, Elekta). The trajectory, angle and depth of the pituitary gland were calculated. Stereotactic sideplanes were taken off the localizer box, and an arc based stereotaxic frame was mounted, converting it into a stereotaxic arc-based frame with micromanipulator (Bjarkam et al., 2009).

Local analgesia of the epidermis and dermis in the midline on the minipig's forehead was obtained by infiltration of 2.5 ml 5% lidocaine (Lidocain "SAD"). A midline incision to the bone (4–6 cm) was placed guided by the stereotactic arch, before a craniectomy was made using a high-speed electrical drill (Midas Rex, Medtronic). Dura was gently opened using a size 11 scalpel and a custom-made tube (5 mm in diameter) with a blunt stylet was inserted between the frontal lobes, avoiding lesions to the large blood vessels and between the two hypothalami on the calculated trajectory to the pituitary gland. When the pituitary gland was reached in sella turcica, the stylet was removed, a monopolar electrode (Medtronic) was inserted and a 30 s coagulation impulse was fired twice (1 A and 30 V, Maxireg 762, Weir Electronics). Electric potential and current was chosen based on previous pilot egg-yolk and cadaver experiments. To ensure adequate haemostasis, the tube stayed in place for 5 min after the last coagulation impulse. If signs of bleeding would appear (blood in the tube), a 10 s coagulation impulse would have been repeated until haemostasis, however, no bleeding was noted. The tube was removed and the skin was closed using one-layer single dissolvable sutures (Vicryl 2–0, Ethicon). Post-operative analgesia consisted of 1 ml buprenorphine (Temgesic®) (0.01 mg/kg) IM and flunixinmeglumin (Finadyne® vet)(2.2 mg/kg). Procainpenicillin (Penovet) 300.000 IE/ml (3.5 ml per 50 kg) was used as postoperative antibiotic treatment. Animals were postoperatively re-scanned using same method as previously to visualize the coagulation and any adverse effects. After MRI-scanning the inhalation anaesthesia was stopped, and the pigs were allowed to wake up. The intubation tube was removed at the first observed gag reflex and the pigs were transferred to an observation pen, until standing upright and starting eating. After the surgical procedure, the animals were transferred to the stables for pain relief treatment, general recovery observation and further handling. Pain relief treatment in addition to the treatment given during anaesthesia consisted of buprenorfin (Vetergesic) (0.01 mg/kg) three times and meloxicam (Metacam) (0.02 ml/kg) one time daily for 4 days. Signs of side effects from the surgery were expected to include impaired sight, blindness or other signs of brain damage. However, no signs of side effects were noted and the pigs began eating immediately after recovering from anaesthesia.

2.4. Hormonal replacement therapy

As the entire pituitary gland was expected to be affected by the surgery, other pituitary hormones than GH were considered for replacement therapy to ensure animal welfare after the surgery (Prabhakar and Shalet, 2006; Veldhuis, 2013). The lack of prolactin, follicle-stimulating hormone, luteinizing hormone or oxytocin was not expected to affect welfare within 7 days. Lack of adrenocorticotropic hormone, thyroid-stimulating hormone and anti-diuretic hormone could induce discomfort if abolished by the surgery, thus, the minipig received 25 mg cortisol and 3 µg desmopressin daily as well as 0.25 mg hydro-thyroxine twice weekly. This was done without verifying the need, based on the experimental and animal welfare protocol.

2.5. Evaluation of surgery – diagnostic testing

To be able to infuse drugs and obtain blood samples in a stress free manner during the diagnostic procedure, a semi-permanent catheter was placed. The catheters were inserted into the jugular vein through the ear vein (Careflow without extension 20G, 20cm Argon ref.681644.) under sedation one week prior to pituitary surgery as described (Larsen et al., 2002). Furthermore oral meloxicam (Metacam) administered in the food (0.02 ml/kg, 24 h coverage) was administered once daily for 3 days. The catheters were flushed with sterile saline daily through-out the study. The insulin tolerance test (ITT), the golden standard of GH deficiency diagnostic testing in humans, was used to evaluate the surgical procedure. Measure of GH content in plasma without a prior challenge is not sufficient to diagnose GHD, as GH is secreted in a pulsatile manner, in particular during night time (Gill et al., 1999; Kargi and Merriam, 2013). Also, IGF-1 plasma concentrations in a blood sample are unreliable as diagnostic tool as there can be an overlap between the IGF-1 levels of GHD patients and healthy controls (Fukuda et al., 2014). Insulin-induced hypoglycaemia results in increased stimulation of GH secretion from the pituitary gland and GH can be detected in blood samples obtained frequently for 90 min after insulin administration. The ITT was performed after an overnight fast with *ad libitum* access to water. A bolus of 0.6 nmol/kg insulin (Actrapid, Novo Nordisk, Denmark) was given IV and blood samples were obtained at -5, 5, 10, 20, 30, 40, 50, 60, 75 and 90 min after dosing for determination of GH, insulin and glucose concentrations as described below.

Blood samples:

Whole blood was placed in 8 mM EDTA-coated tubes on ice until centrifugation at 4000 rpm, 4 °C for 10 min for plasma collection. Glucose concentrations were measured in whole blood using EKF Biosen Autoanalyser (EKF diagnostic GmbH, Barleben, Germany). Insulin, GH and IGF-1 plasma concentrations were determined by beads-based luminescence oxygen channeling immuno assays as described in (Poulsen and Jensen, 2007; Thorsted et al., 2016; Ullman et al., 1996).

The blood glucose concentrations were monitored during the test by hand-held devices (Accu-chek, Roche, Denmark) to monitor blood glucose concentration and diagnose hypoglycaemia. The pigs were constantly and carefully observed for clinical signs of hypoglycaemic shock such as shaking, vomiting and loss of continuousness. Mild to moderate hypoglycaemia indicated by search for food in the bedding material and drooling, was considered acceptable without any intervention. However, no clinical signs of hypoglycemic shock or treatment-requiring hypoglycemia were observed.

2.6. Histology

Brains and pituitary gland were removed as previously described (Bjarkam et al., 2017).The pituitary regions were fixed by immersion in a 4% paraformaldehyde solution (pH 7.4) for 24 h, placed in 30% sucrose solution for 72 h, and frozen in isopentane cooled with dry ice. The sample was kept frozen during cryosectioning into 40 µm horizontal sections (except for pig 2, which was sectioned sagittally, *see* Fig. 3B2) and directly mounted on microscopic slides. Finally, the sections were stained with haematoxylin and eosin, dehydrated in alcohol and xylene, mounted with Depex, and coverslipped.
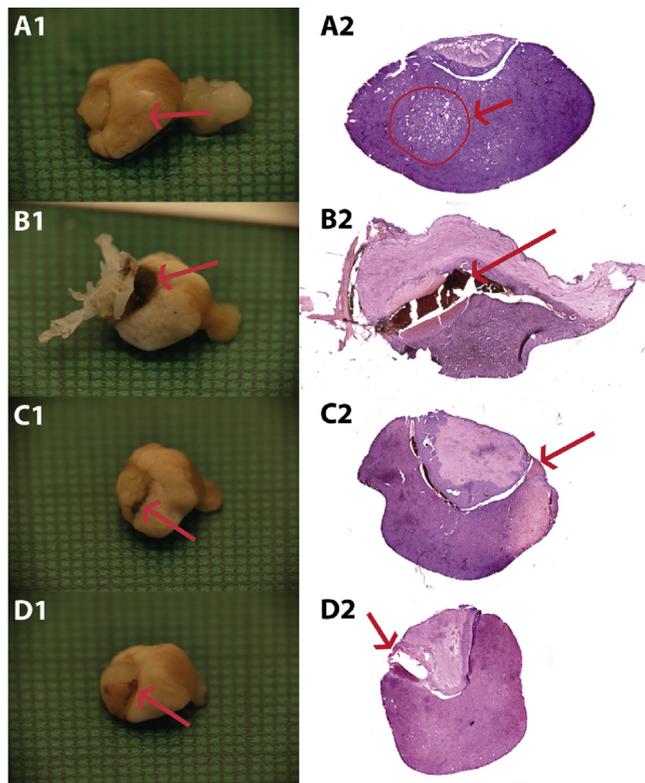
Fig. 3. Macroscopic and microscopic pictures of the pituitary gland after removal from sella turcica. The damaged tissue is marked with red arrows. Left panel: Pictures with the number 1 show the macroscopic Right Panel: Pictures with the number 2 are pictures of histological sections. Pictures labelled A are from pig 1, B are from pig 2, C are from pig 3, and D are from pig 4.

2.7. Statistical considerations

The current study is a proof-of-concept study. Four pigs were subjected to the procedure without sham-operated controls. GH and IGF-1 concentrations were assessed before and after surgery.

3. Results

3.1. Magnetic resonance imaging scans before and after surgery

MR images were obtained before and after the pituitary surgery and a representative image is seen in Fig. 4. The images suggested a successful surgery as the bright signal from the pituitary gland and surrounding blood vessels in the image to the left (blue arrow) was diminished in the post-surgical image on the right (blue arrow).

3.2. Circulating levels of insulin, glucose and GH during ITTs

In the ITT, the elimination of insulin was initiated immediately after administration and completed after about 30 min. High insulin levels caused plasma glucose levels to drop to about 2 mM 10–30 min after administration of insulin.

GH secretion was detected in all pigs with maximum at 30–50 min after insulin administration in the ITT before (Fig. 5A) and after (Fig. 5B) surgery.

Our study did not show statistical differences between GH AUC before and after surgery. Pig 1 to 3 had unaffected or increased GH AUC after surgery (Fig. 6A), while pig 4 have decreased GH levels compared to before surgery. Mean IGF-1 concentrations before and after surgery was not statistically different. IGF-1 concentrations were decreased in pigs 1–3 and increased in pig 4 (Fig. 6B).

The hormonal replacement treatment during the week after surgery was successfully tolerated via functioning IV catheters and pieces of apple with embedded oral tablets.

3.3. Histological evaluation

The macroscopic evaluation of the pituitary tissue prior to histological processing revealed that the pituitaries were not completely abolished by the electrocoagulation but clearly showed that all four pituitaries were targeted by the electrocoagulation; pig 1 had no evident burn on the surface, however, the surface was clearly scared by the electrocoagulation. Pig 2, pig 3, and pig 4 showed obvious burns from the electrocoagulation on the surface (*see* Figs. 3A1-D1 and 7A-D).

The histological sections showed that for all four pituitaries, the tissue was accurately targeted, and the effect of the electrocoagulation could be seen also on the microscopic level (*see* Fig. 3A2-D2). Histological sections from pig 1 showed only small defects in the tissue (altered area in the centre and coagulated spots). Sections from pig 2 showed significant
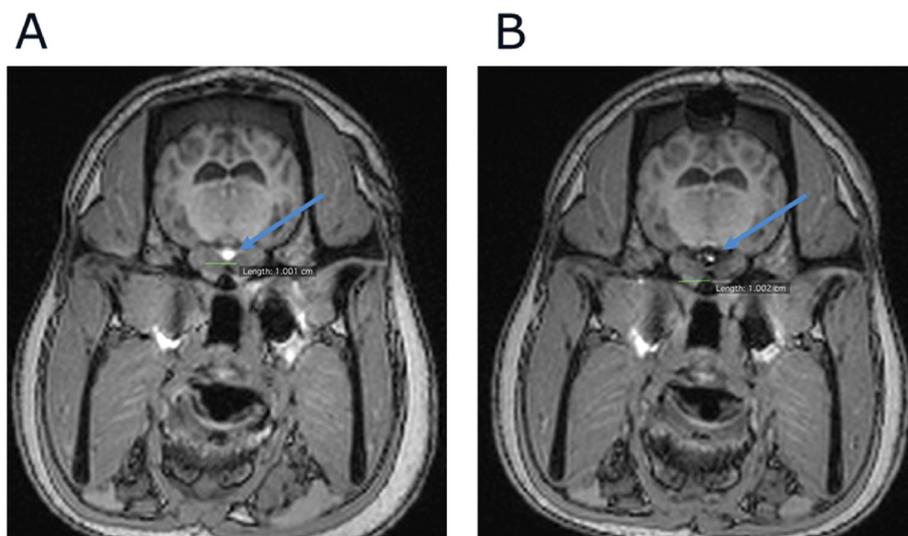

Fig. 4. MR images of a representative pig before (**A**) and after (**B**) pituitary surgery.
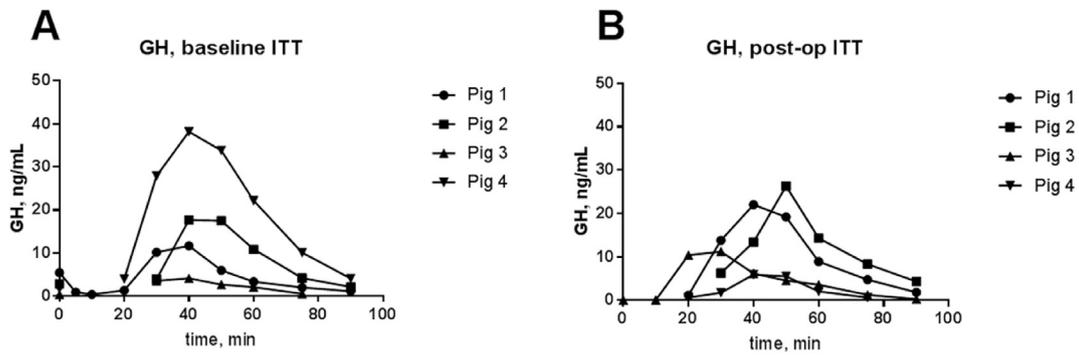
Fig. 5. GH plasma concentrations levels duing ITT before (A) and after (B) surgery.
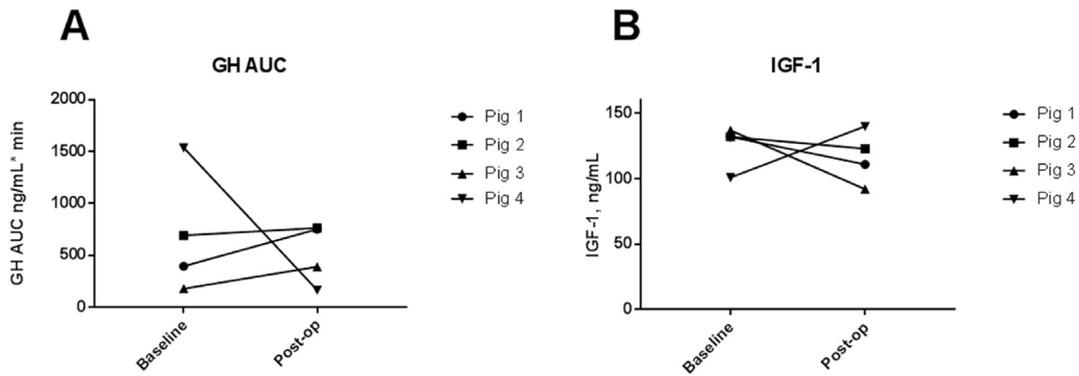

Fig. 6. Area under the curve of GH during the ITT and IGF-1 concentrations before and after surgery. A: Area under the curve (AUC) GH B: IGF-1 concentrations before and after surgery.
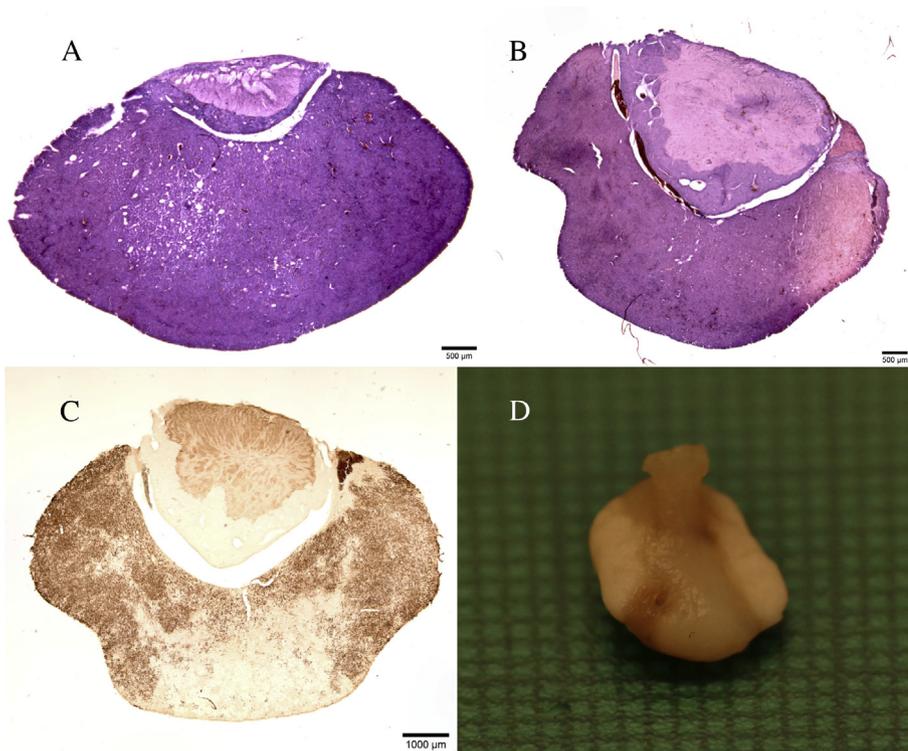

Fig. 7. Higher magnification of pituitary gland with examples for evaluation. A: Visualizing vacuoles and erythrocytes trapped after damage from electro-coagulation. B: At the right hand side of the picture, discolorations at the area of entrance of the electro-coagulation probe. Haematoma is seen between the adeno and neuro lobes. C: Corresponding slide to B, but stained for growth hormone. Again at top right hand side, a small haematoma at the entrance of electrocoagulation probe, and qualitative signs of discoloration near the entrance of electrocoagulation probe. For D: scale bar 1 mm.

damage to the central portion of the gland across the entire length of the border between the anterior and posterior lobe of the pituitary. Pig 3 had a damaged area in the most posterolateral part of the anterior lobe near the border to the posterior lobe. Pig 4 was targeted in the lateral part of the border between the anterior and posterior lobe (see Fig. 3A2-D2 and 7A-D).
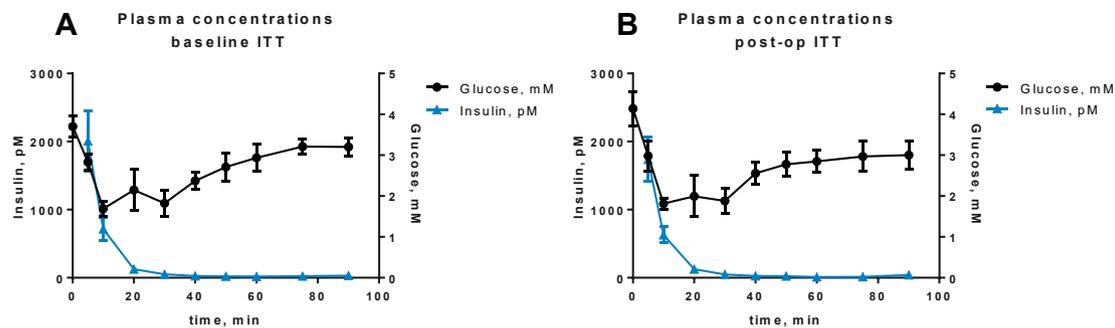
Fig. 8. Plasma levels of glucose and insulin during ITTs before (**A**) and after (**B**) surgery. Insulin (blue triangles) on the left y-axis and glucose (black dots) on the right y-axis.

4. Discussion

The aim of the present study was to evaluate methods for a large animal model of AGHD.

The MRI scans show that the precise targeting of the stereotaxic frame system was impeccable and successfully avoided vital brain structures ensuring the long-term survival of the pigs. The targeted area found visible on the MRI scans seemed to implicate affection to most of the pituitary volume. However, the histological sections (Fig. 3) revealed that the applied electrical current was inadequate to eliminate the entire pituitary gland, as desired. The size of the obtained hypodense area on the post operative MRI-scans that presumably corresponded to damage of tissue might be overestimated due to blood coagulates and oedema in surrounding tissue. It can be speculated that other MRI sequences for the postoperative scan might constitute a more reliable evaluation of the effect of electrocoagulation if it would be possible to distinguish between tissue destruction, blood coagulates, and oedema. Another proposition for future studies is the use of another translational-model modality than electrocoagulation for the tissue destruction such as the gamma knife LINEAC, radio frequency ablation or proton beam radiosurgery. The technique enables a precise preoperative dose planning and delivers highly focused and intense beams of radiation to the target with great ability to spare surrounding tissue. An alternative modality could also be a catheter that can be introduced via the micromanipulator arm and then inflated to exert mass effect on the pituitary. Furthermore, gene transfer via e.g. AAV-vectors and gene-lowering might be an option in future models. In this setup, we chose the electrocoagulation due to its availability for the research group. With future anaesthetics with little or no interference on the metabolism, it might even be possible to perform continuous physiological test, while manipulating the pituitary gland.

During the ITT, blood levels of insulin and glucose were affected as expected (Fig. 8) (Yuen et al., 2016). Insulin administration resulted in hypoglycemia, which stimulated release of GH from the pituitary gland. GH secretion was detected in all pigs with maximum at 30–50 min after insulin administration indicating that insulin-induced hypoglycemia stimulated release of GH in pigs and that the diagnostic tests were successful (Fig. 5).

Although not visible on histological sections, hormone-producing function could be affected by the electrocoagulation. Thus, it could be speculated that induced cell damage that was not visible on the histological sections may have impaired hormone-producing function more than expected from the histological evaluation. However, the insulin tolerance test showed that GH AUC was unaffected or increased in pig 1–3, while it was decreased in pig 4 after surgery compared to before surgery (Fig. 5A). This could be interpreted as unsuccessful surgeries in pig 1–3 and successful surgery in pig 4, but the GH AUC after surgery in pig 4 may not be different from the baseline GH AUC of pig 3 and could thus be in the normal range. However, histological evaluation showed no substantial damage to pig 4 compared to the other animals. Further

evaluation of the histological architecture of the minipig pituitary might shed light on the optimal placement of the coagulation in order to target specific sub-population of cells. Additional changes on the GH-IGF-I axis might be detected in a setup with a longer post-operative evaluation period.

Electrocoagulation was expected to cause impairment of GH secretion and reduced hepatic IGF-1 secretion (Taheri et al., 2014). However, the one week observation period after surgery is not considered to be sufficient to wash out IGF-1 secreted before surgery, and IGF-1 would not be optimal to evaluate the surgical procedure as it can also be affected by other factors ($t^{1/2}$ for IGF-J, 14–18 h) (Bielohuby et al., 2011; Clemmons, 2012). IGF-1 concentrations before and after surgery were not statistically different.

The hormonal replacement therapy was successfully tolerated and no side effects were observed. As the surgery did not result in pituitary dysfunction, it cannot be determined if the hormone replacement would have been sufficient. Our approach might not be totally satisfactory based on a final model, but a lot of the pitfalls and obstacles have been overcome. The model setup with surgical approach and biochemical evaluation might open up to other angles of AGHD-modelling, especially with stereotaxic approaches.

5. Conclusions and perspectives

The capability of the minipigs to secrete GH during ITT was not impaired by surgery. The location of and insult to the pituitary gland was precise. Although the coagulation was apparently too gentle, that is by far preferred over to large ablation, due to the risk of bleeding and the possible damage to adjacent brain areas including the hypothalamus, the optic chiasm, the internal carotid arteries, and the basal forebrain. An important aspect for future application of this method as a translational model of pituitary disease is the long-time survival of the animals and the fact that all included animals recovered well and survived until termination of the study.

The diagnostic test, ITT, successfully induced GH release from the pituitary gland and consequently, the golden standard of diagnostic testing for GHD in humans has been translated to a porcine model. During the ITT, the minipigs were affected by hypoglycaemia, but no cases of hypoglycemic shock were observed. Hormone replacement therapy with cortisol, desmopressin and thyroxin was administered and did not cause side effects. The protocol for intubation, anaesthesia and analgesia for the surgery, was successful, and the minipig's natural behaviour patterns before and after surgery did not change.

In summary — although unsuccessful in eliminating GH secretion — the experimental setup allowed generation of a relevant platform for a model of adult onset GHD in humans, in terms of diagnostic test, precise targeting of the pituitary gland using MRI-guided stereotactic localization, hormonal replacement therapy and animal analgesia, anaesthesia and animal welfare. To generate an adult onset GHD model in minipig, a

study of radio frequency ablation as a method to cause dysfunctional pituitary tissue could be performed. Once a method to induce dysfunction of the pituitary tissue is developed, the animal model should be characterised in terms of body composition, glucose tolerance, bone mineral density, bone mineral content and others, e.g. development of atherosclerosis and possibly cognitive function. Furthermore, the response to GH administration should be characterised to evaluate the minipig as a model of adult onset GHD.

Declarations

Author contribution statement

Laura Hvidsten Ørstrup Andreas Nørgaard Glud: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Laura Tvilling, Dariusz Orlowski: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Hamed Zaer: Performed the experiments.

Carsten Reidies Bjarkam: Analyzed and interpreted the data; Wrote the paper.

Pia von Voss, Pia Skårup Andersen, Berit ø. Christoffersen, Jens Christian Hedemann Sørensen Torben Laursen, Peter Thygesen, Jens Lykkesfeldt: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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