Study Title: Effect of intranasal insulin on LH concentrations in man			
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ABSTRACT

Studies over the last few years have clearly established that at least 25% of men with type 2 diabetes have subnormal free testosterone (T) concentrations in association with inappropriately low LH and FSH concentrations. These patients thus suffer from hypogonadotrophic hypogonadism (HH). Obesity and metabolic syndrome are also associated with HH. Animal studies and in vitro data have shown that insulin action and insulin responsiveness in the brain are necessary for the maintenance of the functional integrity of the hypothalamo-hypophyseal-gonadal axis. Insulin concentrations can be increased in the brain by delivering insulin intranasally with vianase device. The aim of this project is to study the effect of one dose of intranasal insulin on release of LH in diabetic men and healthy menwith a normal BMI. 14 men with type 2 diabetes and 14 healthy men will be recruited for the study. The study will be carried out at the clinical center of TTUHSC-PB (Odessa campus). There will be two study visits, each comprising of blood tests every 15 minutes over 5 hours. 40 IU of intranasal insulin or saline will be given after a baseline period of blood sampling of 2 hours. Student's *t*-test will be used to compare the change in LH concentrations after intranasal saline or insulin.

BACKGROUND

Studies over the last few years have clearly established that at least 25% of men with type 2 diabetes have subnormal free testosterone (T) concentrations in association with inappropriately low LH and FSH concentrations. These patients thus suffer from hypogonadotrophic hypogonadism (HH). Another 4% have subnormal testosterone concentrations with elevated LH and FSH concentrations. Obesity and metabolic syndrome are also associated with HH.

Association of type 2 diabetes, obesity and metabolic syndrome with low testosterone

Subnormal free testosterone concentrations in association with inappropriately low LH and FSH concentrations in men with type 2 diabetes were first described in 2004(1). These abnormalities were independent of the duration and severity of hyperglycemia (HbA1c). Magnetic resonance imaging in these hypogonadal patients showed no abnormality in brain or the pituitary(1). The response of LH and FSH to GnRH injection was normal. It has now been shown that kisspeptin injection also leads to an increase in gonadotropin and T concentrations in men with type 2 diabetes(2) (discussed later in more detail).

This association of HH with type 2 diabetes has now been confirmed in several studies and is present in 25-40% of these men (3-6). In this context, it is important that the Endocrine Society now includes type 2 diabetes in the list of conditions known to have a high prevalence of hypogonadism and suggests screening men with type 2 diabetes for hypogonadism(7). Younger patients with type 2 diabetes (between the ages of 18 and 35 years) also have a similarly high prevalence of HH(8). In all of the above studies, total T and free T concentrations were inversely related to BMI and age. However, the presence of low T concentration was

not entirely dependent upon obesity since 25% of non-obese patients(31% of lean and 21% of overweight) also had HH(1). HH is relatively rare in type 1 diabetes, and, therefore, is not a function of diabetes or hyperglycemia per se(9). Thus, in view of the inverse relationship between BMI and T concentrations in both type 1 and type 2 diabetes, HH is probably related to insulin resistance (1, 5, 9). Previous studies have shown that hypogonadism is associated with upper abdominal adiposity, insulin resistance and the metabolic syndrome (10, 11). We presented preliminary data from a study comparing the insulin resistance in type 2 diabetic men with subnormal or normal T concentrations at the annual meetings of the ADA, the Endocrine Society and AACE this year (12). 81 men with type 2 diabetes had their insulin sensitivities measured by hyperinsulinemic euglycemic clamps. 39 men had HH and 42 were eugonadal. Men with HH had greater BMI (39 vs 34 kg/m^2, p=0.002) and fat mass (46 vs 34 kg, p<0.001) but similar age (54 vs. 52 years, p=0.4) and HbA1c (7.1 vs. 7.2%, p=0.5) as compared to eugonadal men. The glucose infusion rate was 30% lower in men with HH as compared to eugonadal men, even after adjustment for the difference in fat mass among the two groups (p=0.02). Treatment of systemic insulin resistance by rosiglitazone leads to a modest increase in T concentrations in men with type 2 diabetes(13), without the restoration of T concentrations to normal.

Many studies have shown free T concentrations to be also low in the obese, especially in those with BMI≥40kg/m^2.(14, 15) Vermeulen et al (15) compared 35 obese men (mean BMI 41.1 kg/m^2) with 54 lean men. The FT concentrations were 26% lower in the obese. The Free T concentrations correlated inversely with BMI. The authors also compared LH pulsatility over 12 hours in 8 obese and lean men and found that the mean integrated LH levels over 12 hours were significantly lower in obese men. Free T concentrations correlated positively with the sum of LH pulse amplitudes in each individual(15). A study from Netherlands in 160 obese men (mean age 58 years) found a 35.6% prevalence of HH (16). Nielsen et al(17) examined T concentrations of 615 non-obese and 70 obese young Danish men (20-29 years of age) in association with subcutaneous and visceral fat mass, measured by DEXA and MRI. Total and bioavailable T concentrations were negatively related to all measures of fat mass. 23% of obese young men had subnormal total T concentrations, while 10% had subnormal total and bioavailable T concentrations. The largest study in this regard compared the prevalence of low testosterone concentrations in obese and diabetic men (mean age 60 years; range: 45-96 years)(18): 44% of diabetic and 33% of age matched non-diabetic men had subnormal free testosterone concentrations respectively. 40% of obese men and 50% of obese diabetic men had subnormal free testosterone concentrations. Thus, obesity is associated with a high prevalence of hypogonadism and the presence of diabetes adds to that risk. However, this study was restricted to a relative elderly population (age >45 years). Furthermore, these studies did not utilize the current gold standard method of measuring testosterone, liquid chromatography tandem mass spectrometry (LC-MS/MS). Therefore the actual prevalence of hypogonadism in obesity may be lower.

We have recently studied the impact of obesity on T concentrations in boys at the completion of puberty(19). We compared the T, LH and FSH concentrations of 25 lean and 25 obese boys in tanner stage 4 and 5. The free T concentrations (measured by equilibrium dialysis followed by LC-MS/MS) of obese boys were 40% lower than those of lean boys. The gonadotropin concentrations of lean and obese boys were similar. The T concentrations were inversely related to BMI, HOMA-IR (homeostatic model assessment- insulin resistance) and to C-reactive protein concentrations.

Similar to the negative impact of obesity on T concentrations in men, studies have highlighted the association of metabolic syndrome(MetS) with hypogonadism(20). Kaplan et al studied 864 men (mean age 52 yrs) and demonstrated that aging men with obesity and the MetS have a significantly decreased total T (150 and 300 ng/dl less in obese and severely obese respectively) compared to aging, metabolically healthy men(21). Free T concentrations decrease proportionately with increasing number of components of metabolic syndrome(22). In a population based study of 1896 middle aged Finnish males (345 of whom had MetS), Laaksonen et al showed that Free T and SHBG were respectively 11%

and 18% lower in MetS than in normal subjects (11). Men with Free T in the lowest third tertile were 1.7 times (after adjustment for age and BMI) more likely to have MetS. Low T and low SHBG concentrations were associated with MetS and its components, independently of BMI. Total T, Free T and SHBG were inversely associated with concentrations of insulin, glucose, triglycerides and CRP, and positively associated with HDL. Obesity might be the most important contributor to the presence of hypogonadism in MetS, and the linear inverse relationship of T with BMI is preserved in the presence of MetS(8; 10). Thus from the preceding discussion, it appears that insulin resistance in men is associated with HH.

Possible pathophysiological mechanisms underlying HH in type 2 diabetes/obesity

a) Role of Estradiol: Since testosterone and androstenedione in the male can be converted to estradiol and estrone respectively through the action of aromatase in the mesenchymal cells and pre-adipocytes of adipose tissue, it has been suggested that excessive estrogen secretion due to aromatase activity in the obese may potentially suppress the hypothalamic secretion of GnRH(23). It therefore follows that the oestradiol concentrations in men with HH and type 2 diabetes /obesity should be elevated to account for the suppression of gonadotropin secretion. However, we have shown that this widely believed presumption is not true(24). We measured the estradiol concentrations in 240 type 2 diabetic men with and without HH. Total estradiol concentrations were measured by immunoassay and free estradiol concentrations were calculated, using SHBG. Total and free estradiol concentrations in men with HH were significantly lower than in those without HH(24). To confirm these findings, total estradiol concentrations were measured in a subset of 102 men by the LC-MS/MS assay and free estradiol concentrations were measured by equilibrium dialysis (24). Estradiol concentrations were 25% lower in men with HH. Free estradiol concentrations were directly related free T concentrations, irrespective of age or BMI. As mentioned above, our recent data on young pubertal and post-pubertal males (14-20 years of age) have shown that free testosterone concentrations are approximately 40% lower in obese adolescents as compared to lean adolescents(19). Similar to our study in middle-aged men with type 2 diabetes, we found that estradiol concentrations were also significantly lower in adolescents with lower free testosterone as compared to those with normal T. The estradiol concentrations related directly to testosterone concentrations but not to BMI. Population based studies such as European Male Aging Study also showed that estradiol concentrations are lower in hypogonadal men as compared to eugonadal men, regardless of whether the hypogonadism is primary or secondary (25). In fact, no study has shown increased concentrations on estradiol in hypogonadal men as compared to eugonadal men with obesity or type 2 diabetes. The direct relationship between plasma estradiol and T concentrations is consistent with the concept that estradiol concentrations are dependent upon testosterone, the substrate for estradiol synthesis. Clomiphene (estrogen antagonist) and aromatase inhibitors (which decrease estradiol concentrations) have been shown to increase testosterone concentrations in obese men with low testosterone(26). This, however, cannot be taken as evidence of estradiol as the cause of low testosterone in those men because eugonadal men also respond to aromatase inhibition by an increase in testosterone concentrations. This is because estradiol has an inhibitory effect upon gonadotropin secretion even in a normal physiological setting. A decrease in estradiol action or concentration would lower its inhibitory effect upon gonadotropins and eugonadal men may achieve even supranormal LH, FSH and T concentrations (27). Elevated gonadotropin concentrations are also seen in men with congenital aromatase deficiency or absence of estrogen receptor-α (28, 29). Clearly, the suppression of LH, FSH and T in type 2 diabetes and obesity is not induced by circulating estradiol concentrations.

b) Role of kisspeptin: It is now known that kisspeptin, a hypothalamic neuropeptide encoded by the KISS1 gene, and the presence of kisspeptin receptors on the GnRH neurons (G protein-coupled receptor

54) is obligate for the release of GnRH(30, 31). Humans with absence of either kisspeptin gene or its receptor (GPR54) have HH(31, 32). Data from animal studies show that factors associated with decreased GnRH secretion, such as inflammation and estrogen, decrease KISS1 gene expression in the hypothalamus(33, 34). Intravenous administration of kisspeptin increases LH and testosterone concentrations in men with type 2 diabetes and HH(2), thus suggesting that the hypothalamic-pituitary-gonadal axis is intact *per se* in men with HH and type 2 diabetes. However, the (presumably) metabolic insult in insulin resistance that results in hypogonadotropism is yet to be defined. Kisspeptin neurons express both leptin and insulin receptors, thus possibly accumulating evidence of metabolic health and translating it into reproductive health.

c) Role of inflammatory mediators: Tumor necrosis factor α and interleukin-1β have been shown to suppress hypothalamic GnRH and LH secretion in experimental animals and *in vitro* (35, 36). It is therefore relevant that C-reactive protein(CRP) concentrations are markedly increased in hypogonadal type 2 diabetic men as compared to men with type 2 diabetes and normal testosterone(6.5 mg/l versus 3.2 mg/l) (37). These data were confirmed by another study from Australia in which the median CRP concentration in type 2 diabetic patients with low total T was 7.7 mg/l as compared to 4.5 mg/l in men with normal testosterone(5). Free T concentrations were inversely related to CRP concentrations. It is thus possible that inflammatory mediators may contribute to the suppression of the hypothalamo-hypophyseal axis and the syndrome of HH in type 2 diabetes. The presence of inflammation may also contribute to insulin resistance since several inflammation related mediators, such as suppressor of cytokine signaling-3, IκB kinase β and c-Jun N-terminal kinase-1 interfere with insulin signal transduction (38, 39) and contribute to insulin resistance. These mediators are also known to be increased in obesity(40).

d) Role of Leptin: Leptin is known to play a permissive role in the regulation of reproductive axis. Leptin appears to serve as a signal of signal of energy reserves to regulate the hypothalamo-pituitary-gonadal axis in relation to nutritional status (41). Men and women with anorexia nervosa have low levels of leptin and hypogonadotropism. Absence of leptin gene or leptin signaling in humans results in HH (42). While there are extremely uncommon forms of human obesity due to lack of leptin gene, almost all obesity in humans is associated with leptin resistance and high leptin concentrations. It is possible that leptin resistance in hypothalamic neurons or in some other neurons is responsible for the hypogonadotropism seen in obesity. Direct evidence in humans supporting or disproving this reasoning is, however, lacking.

e) Role of insulin resistance: The selective deletion of the insulin receptor from neurons leads to a reduction in LH concentrations by 60%-90% and low T concentrations (43). These animals respond to GnRH challenge by normal or supra- normal release of LH. In addition, these animals had atrophic seminiferous tubules with markedly impaired or absent spermatogenesis. In addition, it is known that the incubation of hypothalamic neurons with insulin results in the facilitation of secretion of GnRH(44, 45). Thus, insulin action and insulin responsiveness in the brain are necessary for the maintenance of the functional integrity of the hypothalamo-hypophyseal-gonadal axis. However, it is unlikely that the site of action of insulin on reproductive axis is the GnRH neuron. Isolated knock out of insulin receptor in GnRH neuron does not lead to a decrease in LH concentrations or in fertility in either male or female mice(46). The loss of insulin receptors in proopiomelanocortin- or agouti-related peptide-expressing (POMC- or AgRP-expressing) neurons — neurons involved in the regulation of appetite and peripheral metabolism of glucose and fat — also did not affect fertility(47). However, the selective deletion of insulin receptors from kisspeptin neurons resulted in a delay in puberty in both male and female mice, although LH

concentrations, T concentrations and fertility during adulthood were not affected (48). Thus, the site or sites) of hypogonadtropism seen in neuronal insulin receptor knock-out mice is still not clear.

In summary, it is likely that there are several inter-linked causative mechanisms underlying HH in men with type 2 diabetes. It is possible that delivery of insulin to the hypothalamus may enhance the secretion of LH (probably by increasing kisspeptin stimulated GnRH secretion). The following paragraphs briefly describe the literature on central effects of insulin, with emphasis on human studies. The human experiments have used an intranasal approach to increase insulin levels in the brain.

CNS actions of insulin

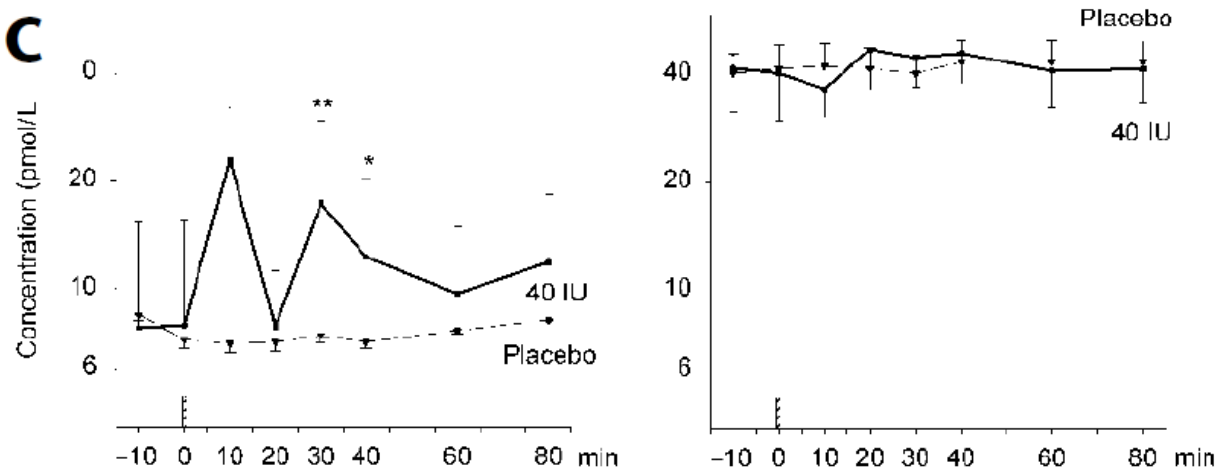
Havrankova et al. showed in 1978 that insulin receptors are present throughout the rat CNS, followed closely by the demonstration that insulin receptors are also expressed in the human brain(49, 50). The insulin receptor is found in particularly high densities in brain regions like the olfactory bulb, the cerebellum, the dentate gyrus, the pyriform cortex, the hippocampus, the choroid plexus and the arcuate nucleus of the hypothalamus. It is assumed that peripheral insulin crosses the blood–brain barrier (BBB) by a saturable, receptor-mediated transport mechanism and by binding to brain insulin receptors affects functions as diverse as energy and glucose homeostasis, reproduction and cognition (43, 51-53). There are good data in rodents that brain insulin signaling suppresses hepatic glucose output (54). Lack of brain insulin receptors leads to mild diabetic phenotype in mice, along with HH as mentioned above (43). Studies also suggest that IN insulin reduces sympathetic outflow to adipose tissue and decrease free fatty acid concentrations(55). There are only a few human studies on intranasal insulin's effects on metabolism. Acute administration of intranasal insulin reduces food intake while chronic administration reduces fat mass (56, 57). These effects are more pronounced in men than in women for unclear reasons. Acute intranasal administration also decreased HOMA-IR(58). A recent study showed that intranasal insulin increased insulin sensitivity measured by hyperinsulinemic euglycemic clamp (gold standard for measuring insulin sensitivity) by 41% in lean men(59). This was accompanied by an increase in activity of parasympathetic nervous system and an increase in cerebral blood flow in the hypothalamic region (as assessed by functional magnetic resonance imaging). There was no change in insulin sensitivity of overweight/obese men. Presumably, there is "brain insulin resistance" in obese men but there is no direct evidence for this. Intranasal insulin has been shown to improve cerebral blood flow, cortical activity, memory and cognition in healthy subjects, diabetics and in patient with Alzhemier's disease(53, 60).

In the proposed project, we will study the effect of intranasal insulin on LH concentrations in lean healthy men as well as in men with type 2 diabetes.

Pharmacokinetics of intranasal (IN) insulin: Born et al measured concentrations of insulin in CSF and serum for 80 minutes after IN administration(61). 27 men and 9 women were studied. After IN dose of 40IU regular insulin, CSF insulin concentrations increased rapidly and remained elevated above baseline for the duration of the study (figure above). The area under curve increased from 603 to 1091pmol/l*min. There was no change in serum insulin concentrations. Results were similar with ACTH or vasopressin administration.

It is assumed that after IN administration, insulin travels extracellularly through patent intercellular clefts in the olfactory epithelium to diffuse into the subarachnoid space. This appears more plausible that the alternate intracellular route which would carry a greater risk of proteolysis and would take hours to reach the olfactory bulb(62).

CSF **Serum**



Vianase device: Vianase is a liquid drug delivery system based on controlled particle dispersion technology. It is manufactured by Kurve technology (www.kurvetech.com). The device is being donated by Kurve technology for this research project. They usually donate one device to a center (to begin with) after a "material service agreement" has been reached. The device has a disposable nose piece and hence can be re-used. The insulin (regular human insulin) is reconstituted for each application. The device delivers a metered dose of insulin into the chamber that covers the subject's nose. Using the principal of vortical flow, Controlled Particle Dispersion effectively disrupts inherent nasal cavity airflows to deliver formulations to the entire nasal cavity. By varying control parameters, CPD can target specific nasal regions including the olfactory region and the paranasal sinuses while minimizing peripheral deposition to the lungs and stomach.

Device Information provided by Kurve Technology, Inc.

ViaNase is a Class I electrically powered atomizer operating under Code CFR 21 and JPW 8774.5220

Device	Pump, nebulizer, electrically powered

Regulation Description	Ear, nose, and throat drug administration device.
Regulation Medical Specialty	Ear Nose & Throat
Review Panel	Ear Nose & Throat
Product Code	JPW
Submission Type	510(K) Exempt
Regulation Number	874.5220[6]
Device Class	1
Total Product Life Cycle (TPLC)	TPLC Product Code Report[7]
GMP Exempt?	Yes

Note: This device is also exempted from the GMP regulation, except for general requirements concerning records (820.180) and complaint files (820.198), *as long as the device is not labeled or otherwise represented as sterile.*

Note: FDA has exempted almost all class I devices (with the exception of reserved devices[8]) from the premarket notification requirement, including those devices that were exempted by final regulation published in the *Federal Registers* of December 7, 1994, and January 16, 1996. It is important to confirm the exempt status and any limitations that apply with 21 CFR Parts 862-892[9]. Limitations of device exemptions are covered under 21 CFR XXX.9, where XXX refers to Parts 862-892.

If a manufacturer's device falls into a generic category of exempted class I devices as defined in 21 CFR Parts 862-892[10], a premarket notification application and FDA clearance is not required before marketing the device in the U.S. however, these manufacturers are required to register their establishment. Please see the Device Registration and Listing website[11] for additional information.

Third Party Review	Not Third Party Eligible

(Continued)

TITLE 21--FOOD AND DRUGS

CHAPTER I--FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH AND HUMAN SERVICES

SUBCHAPTER H--MEDICAL DEVICES

PART 874 -- EAR, NOSE, AND THROAT DEVICES

Subpart F--Therapeutic Devices

Sec. 874.5220 Ear, nose, and throat drug administration device.

(a)*Identification.* An ear, nose, and throat drug administration device is one of a group of ear, nose, and throat devices intended specifically to administer medicinal substances to treat ear, nose, and throat disorders. These instruments include the powder blower, dropper, ear wick, manual nebulizer pump, and nasal inhaler.

(b)*Classification.* Class I (general controls). The device is exempt from the premarket notification procedures in subpart E of part 807 of this chapter subject to the limitations in 874.9. If the device is not labeled or otherwise represented as sterile, it is exempt from the current good manufacturing practice requirements of the quality system regulation in part 820 of this chapter, with the exception of 820.180, with respect to general requirements concerning records, and 820.198, with respect to complaint files.

[51 FR 40389, Nov. 6, 1986, as amended at 59 FR 63009, Dec. 7, 1994; 66 FR 38801, July 25, 2001]

The following are some of the studies that have used the Vianase device to deliver intranasal insulin:
Claxton et al; Sex and ApoE genotype differences in treatment response to two doses of intranasal insulin (20IU or 40IU) in adults with mild cognitive impairment or Alzheimer's disease. J Alzheimers Dis. 2013;35(4):789-97.

Craft et al; Intranasal insulin therapy for Alzheimer disease and amnestic mild cognitive impairment: a pilot clinical trial. Arch Neurol. 2012 Jan;69(1):29-38. Subjects used 10IU bid or 20IU bid for 4 months.

As per authors: "No treatment-related severe adverse events occurred during our study, and most adverse events were minor, such as mild rhinitis. The adverse events that occurred more than 5% of the time in any group are listed in **table":-**

Table 4. Data on Adverse Events and Percentage of Intent-to-Treat Sample for All Adverse Events Occurring for at Least 5% of the Participants in Any Treatment Group

	Treatment Group					
	Placebo		20 IU of Insulin		40 IU of Insulin	
Adverse Event	Events, No.	Sample, %	Events, No.	Sample, %	Events, No.	Sample, %
Light-headedness and/or dizziness	3	10.0	3	8.3	5	13.2
Headache not related to lumbar puncture	1	3.3	4	8.3	2	5.3
Nose bleed	0	0.0	6	8.3	3	2.6
Rhinitis	1	3.3	8	16.7	4	7.9
Upper respiratory tract infection	2	6.7	2	5.6	1	2.6
Fall	2	6.7	1	2.8	1	2.6
Rash	2	6.7	1	2.8	2	2.6
Other	16	46.7	30	58.3	33	60.5
Total	27	56.7	55[a]	72.2	51[b]	68.4

[a] $P < .05$ for comparison of 20-IU dose insulin group vs placebo group.
[b] $P < .10$ for comparison of 40-IU dose insulin group vs placebo group.

Novak et al; Enhancement of Vasoreactivity and Cognition by Intranasal Insulin in Type 2 Diabetes. Diabetes Care. 2013 Oct 25. Dose used was 40IU (given once for the study).

This study is very similar to this protocol because we intend to give patients one dose of 40IU. As per authors, in this study, "there were no hypoglycemic episodes, nasal irritation or allergic reactions to insulin. Glucose levels and vital signs were stable and similar across insulin and placebo conditions in both groups".

Significance: HH is one of the most common conditions associated with insulin resistance state in men. Very little is known about its pathophysiology. This study will attempt to evaluate the effect of increasing insulin concentrations in the brain on release of LH.

It is not the aim of this study to diagnose, cure or treat a disease condition (such as HH). The aim is to understand the role of insulin signaling in brain in the release of LH.

Future directions: Leptin has a permissive role in reproductive axis. Once available, intranasal leptin alone or in combination with insulin also needs to be studied.

Hypothesis: One dose of 40 IU intranasal insulin administration increases serum concentrations of LH in healthy men and in men with type 2 Diabetes.

Objective 1: To measure the concentrations of LH every 15 minutes for 3 hours after administration of 40 IU of intranasal insulin or saline.

STUDY DESIGN AND METHODS:

Study population: 14 men with type 2 diabetes and 14 healthy men with a normal BMIwill be recruited for the study.

Screening Day: After informed consent is obtained, all subjects will complete the following procedures:
1) Medical History
2) Physical Exam
3) Baseline HbA1c (in type 2 diabetic men only). If this been done in the last 6 weeks by patient's health care provider, and the results are available, then they will not be re-drawn. This can be done by finger-prick.

Randomization Method: Subjects who qualify for the study will be assigned a number by a computerized random number generation program (Microsoft office – Excel) and will be randomized to receive either intranasal insulin or saline at study visit 1 (randomized, cross-over design). This number will be used as the study subject ID. A master list of subject name and study ID will be kept under lock and key to protect patient identifiers. For visit 2 subjects who received intranasal insulin will receive intranasal saline and vice versa.

The subjects and key study personnel, including the PI, will be blinded to the treatment. The study coordinators, the study subjects and laboratory personnel will be blinded. The sub investigators (Dr. Chemitiganti or Dr. Bilkis) will be responsible for preparation of the drug for vianase device and hence will not be blinded.

Study visit 1: Subjects will come fasting to the clinical center of TTUHSC-Odessa campus between 8 and 9 AM. For the diabetic group, they will not take their anti-diabetic pills in morning. If they are on long acting insulin in AM, that will be withheld as well. Diabetic subjects can take their anti-diabetic pills and long acting insulin the night before the study. In order to avoid the confounding effects of poorly controlled diabetes, patients with HbA1c >8.5% will be excluded.

- A peripheral intravenous cannula will be placed for intermittent blood draws.
- Blood sample will be drawn every 15 minutes for 2 hours, starting at 9AM.
- At 11AM (after the 2 hour samples have been drawn), subjects will spray 40 IU of insulin or equal volume of saline into their nostril once with the vianase device.
- Blood sugar will be checked just before the administration of insulin/saline, and after 30, 60, 120 and 180 minutes. Blood sugar will be checked in the venous blood that is drawn at all these time points (see next tab).
- Blood sample will be drawn every 15 minutes for the next 3 hours.
- Last blood sample will be collected at 2 PM. IV line will then be removed and subjects will be discharged home.

Study visit 2: *This visit will be scheduled for approximately one week after visit 1*. Visit 2 will not be done sooner than 5 days after visit 1 or later than 2 months. The same procedure will be followed as listed for visit 1. Subjects who received intranasal insulin will receive intranasal saline and vice versa.

About 150ml of blood will be drawn during each study visit.

Inclusion Criteria:

- Males age 18 to 75 years
- Type 2 diabetes OR normal (BMI 19-24.9 kg/m^2) healthy men

Exclusion Criteria:-

- HbA1c>8.5%
- Use of testosterone currently or in the past 4 months
- Use of over the counter health supplements which contain androgens
- Use of corticosteroids or narcotics in the past 3 months
- Coronary event or procedure(myocardial infarction, unstable angina, coronary artery bypass, surgery or coronary angioplasty) in the previous twelve weeks
- Type I Diabetes
- Currently suffering from foot ulcer, significant periodontal disease or any other chronic infectious or inflammatory condition
- Known history of hepatic disease (transaminase > 3 times normal) or cirrhosis
- Known history of renal impairment (defined as GFR<30)
- HIV or Hepatitis C positive status
- Any other life-threatening, non-cardiac disease
- History of untreated severe obstructive sleep apnea(defined as apnea-hypopnea index ≥30)
- currently suffering from symptomatic depression, with or without treatment
- Use of an investigational agent or therapeutic regimen within 30 days of study
- Participation in any other concurrent clinical trial
- Pituitary tumor/damage/ other trophic hormone deficiency

Laboratory measurements

Since LH is secreted in a pulsatile manner, LH concentrations will be measured at 15 minute intervals throughout the study (for about 5 hours). The 2 hour period prior to intranasal treatment will serve as the baseline period. Normal men secrete LH pulses at the rate of 0.8 pulses/hr(63). It is likely that the secretion in our study population (with HH) will be lower than normal men.

Apart from the lab tests mentioned above, 10ml of blood will be drawn at time 0h, 2h and 5h and stored for future analysis. We will consider measuring testosterone concentrations in the stored samples if the study shows a rise in LH concentrations.

Statistical analysis: The primary endpoint of the study is to compare the LH concentrations after intranasal insulin as compared to placebo (intranasal saline). Area under curve (AUC) of LH concentrations in the 3 hours following intervention will be compared by paired *t*-test or appropriate non-parametric equivalent. Type I error (α) will be set at 0.05 and Type II error (β) at 0.2. Assuming that there will be a 50% increase in LH concentrations, 12 subjects will be needed for the experiment

provided that the standard deviation of the residuals is no more than the mean change. Assuming a dropout rate of 15%, 14 patients will be recruited. 14 healthy men will also be recruited.

Secondary end points: A secondary endpoint of the study is to compare the change in LH concentrations after intranasal insulin as compared to placebo (intranasal saline). Change will be defined as the difference between baseline LH concentrations (average of the 9 samples collected in the two hours prior to intervention) and average LH concentrations following intervention (average of the 16 samples collected in the four hours following intervention). Paired *t*-test or the appropriate non-parametric equivalent will be used for comparisons. Another secondary end-point will be comparing the change in LH concentrations by repeated measures ANOVA. Finally, we will compare the change in LH concentrations between diabetic men and healthy men.

Method of identifying and recruiting subjects: Subjects will be recruited from the TTUHSC clinics.

Compensation given to subjects: Subjects will receive $200 for completing the study. In case they complete only one study visit, they will receive $100. They will not be compensated for screening visits.

Describe the site(s) where study will be done: The study will be conducted at TTUHSC Odessa campus. Blood tests will be analyzed by LabCorp.

Risks: Placement of IV cannulas may result in pain, a feeling of faintness, irritation of the vein, bruising, or bleeding and possible infection at the site of puncture. Systemic absorption of intranasal insulin is not expected at the dose used in the study, hence hypoglycemia is not anticipated and blood sugars will not be monitored intensively. However, a fingerstick blood sugar will be checked 30 minutes after taking the insulin/saline. Subjects will also be monitored for any symptoms suggestive of hypoglycemia. If there are symptoms at any time, fingerstick blood glucose will be checked. If finger stick is below 60, orange juice will be provided. Patient will then be rechecked in 15 minutes. If still below 60, the patient will be given orange juice and the study will be terminated. Data collected till that time will be stored for analysis.

About 150ml of blood will be drawn during each study visit; this amount is not typically associated with adverse effects.

Although the vianase device is not approved to market, the device and the dose of IH intranasal insulin chosen for this study has been shown to be safe in numerous prior studies which have been referenced in this protocol. It is not the aim of this study to diagnose, cure or treat a disease condition (such as HH). The aim is to understand the role of insulin signaling in brain in the release of LH.

Possible benefits to subjects: Subjects may experience the altruistic benefits of participating in research. They will not have any direct benefit from the study.

Confidentiality measures: All subjects who qualify and agree to be in the study will receive a randomization number. They will then be identified during data collection by their initials and randomization number only. Data will be stored in locked office and password protected computer.

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