DOI:10.14753/SE.2016.1887

DISTRIBUTION OF NOCICEPTIN IN THE PANCREAS AND UTERUS OF NORMAL AND DIABETIC RATS

PhD Thesis

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Budapest

2015

DOI:10.14753/SE.2016.1887

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DOI:10.14753/SE.2016.1887

DOI:10.14753/SE.2016.1887

DOI:10.14753/SE.2016.1887

DOI:10.14753/SE.2016.1887

List of Abbreviations

ABC	Avidin biotin complex
CMHS	College of Medicine and Health Sciences
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAB	3, 3-diaminobenzidine tetrahydrochloride
dL	Deciliter
DM	Diabetes mellitus
EM	Electron microscopy
FFA	Free fatty acid
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDM	Gestational diabetes mellitus
GPCRs	G-protein coupled receptors
HCS	Chorionic somatomammotropin
hPGH	Human placental growth hormone
HPLC	High performance liquid chromatography
i.p	Intraperitoneal
IAPPa	Insulin and islet amyloid polypeptide or amylin
IDDM	Insulin-dependent diabetes mellitus
IEM	Immunoelectron microscopy

mg/rat	milligram per rat
MOP	mu opioid receptors
N/OFQ	Nociceptin/Orphanin FQ
NC	Nociceptin
NE	Nuclear envelope
NMDA	N-Methyl-D-aspartate
NOP	Nociceptin receptor
NOP1	Nociceptin receptor-1
OFQ	Orphanin FQ
OP4	Opioid receptor-4
ORL-1	Opioid receptor-like1
PBS	Phosphate buffer saline
PNS	Peripheral nervous system
PP-cells	Pancreatic polypeptide cells
PPNOC	Prepronociceptin
RIPA	Radioimmunoprecipitation assay buffer
STZ	Streptozotocin
TBST	Tris Buffered Saline with Tween® 20
TEM	Transmission electron microscopy
TNFa	Tumor necrosis factor alpha
TRITC	Tetramethyl rhodamine isothiocyanate

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1. Introduction

1.0 Neuropeptides

Neuropeptides are small molecules used by neurons for extracellular signalling. There are a wide variety of neuropeptides depending upon the required brain function and body physiology. For instance, neuropeptides are involved in analgesia, reward, food intake, metabolism, reproduction, social behaviour, learning and memory [1, 2]. Examples of neuropeptides include enkephalin, dynorphin, nociceptin, neuropeptide Y, relaxin, gastrin, cortistatin, somatostatin and calcitonin gene related peptide. All of these neuropeptides have different biological functions. Neuropeptides and neurotransmitters are extracellular signalling molecules involved in a variety of physiological functions. Some peptide hormones, such as somatostatin, also act as neuropeptides. They are secreted from neuroendocrine glands and travel through the blood circulation to distant target tissues and organs. Whereas, neuropeptides are mainly secreted from neuronal cells and send their signals to neighbouring cells. Many neuropeptides may also be co-released with other small-molecule neurotransmitters. For example enkaphalin, neuropeptide-Y and galanin coexist with norepinephrine, a clasical neurotransmitter [3]. Neuropeptides have long been known to play a regulatory role in complex behaviours, such as learning, and pain sensation and memory [1, 2]. Additionally, the metabolism of neuropeptide, such as dynorphin, is impaired in a number of neurological diseases like schizophrenia and addiction [4]. In summary neuropeptides can act as either neurohormones neurotransmitters or neuromodulators where they help to maintain physiological homeostasis and influence important physiological functions [4].

1.1 Nociceptin

General information

Nociceptin (NC), also known as orphanin FQ (N/OFQ, is the natural ligand for NC receptor, called opiate receptor-like 1(ORL-1) or non-classical opioid receptor (NOP). NC is an endogenous ligand of G-protein coupled receptor (GPCRs) family. Nociceptin is derivative of prepronociceptin (PPNOC), which is present in a large variety of species [5]. Although, NC is an opioid-related peptide, it binds to its own receptor but does not attach to the classic opioid receptors [6, 7]. This neuropeptide is mainly expressed in neurons of

the central (CNS) and peripheral (PNS) nervous systems. The orphan receptor approach regarding the endogenous ligand of the oGPCR ORL-1 was demonstrated successfully with the discovery of N/OFQ, [7, 8].

Meunier et al. [7] used the term nociceptin for the novel peptide that he discovered based on putative pro-nociceptive properties. Almost in th same period, Reinscheid et al. [8] called this new orphan peptide orphanin FQ, as a ligand of an orphan receptor, whose first and last amino acids are Phe (F) and Gln (Q), respectively.

After 1995, a series of publications have provided detailed descriptions of its pharmacological, physiological and behavioural roles [9–11] and emphasized their biological importance in the body. In addition, the pattern of OFQ/N messenger RNA expression in the CNS has also been reported [11].

1.1.1 Structure of nociceptin

Nociceptin (N/OFQ, NC) is a 17-amino acid peptide (Phe-Gly- Gly- Phe –Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Asn-Gln-NH2) (Figure 1) which displays homology in amino acid sequence with opioid peptides, such as dynorphin A, endorphin and enkephalin. NC and dynorphin A both have 17 amino acids bounded by pairs of basic amino acids which are essential in their assembly from precursors peptides. The other similarity between these two peptides is that they have internal pairs of basic amino acids suggesting the likelihood of further processing. These opioid peptides share an YGGF pattern, where the fifth amino acid is either leucine or methionine (Figure 2). The amino terminal of NC is composed of phenylalanine instead of tyrosine, followed by GGF. As a final point, both peptides contain the same last two amino acids at the carboxyl terminal. NC is generated proteolytically from a larger peptide precursor, preproorphanin in a similar manner to endogenous opioids, which contains additional neuropeptides that may have biological activities [5, 12–16]. The structure of the primary rat and human preproorphanin has been elucidated. Since preproorphanin shares close structural homology to the endogenous opioid peptide precursors like prodynorphin and preproenkephalin, it has been suggested that a synchronized mechanism of evolution may have “alienated” NC from the opioid systems [17–19]. The studies on the regional distribution on preproNC, OP4/NOP receptors and NC mRNA showed that they are highly expressed in various neuronal sites with a

pattern wholly distinct from those of classical opioid peptides and share characteristic structural feature mainly with preprodynorphin.

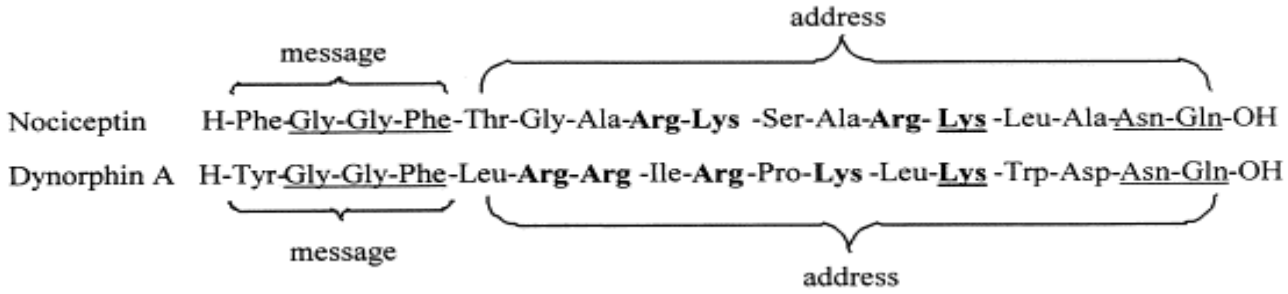


Figure 1: Structural similarities between dynorphin A and nociceptin amino acid sequences [20]**. Message: The domain (N-terminal) responsible for the activation of sequences. Address: The segment (C-terminal) involved in the binding of nociceptin and dynorphin A to specific receptors.**

Nociceptin: Phe-*Gly-Gly-Phe*-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln

Dynorphin A: Tyr-*Gly-Gly-Phe*-Leu-Arg-Arg-lle-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln

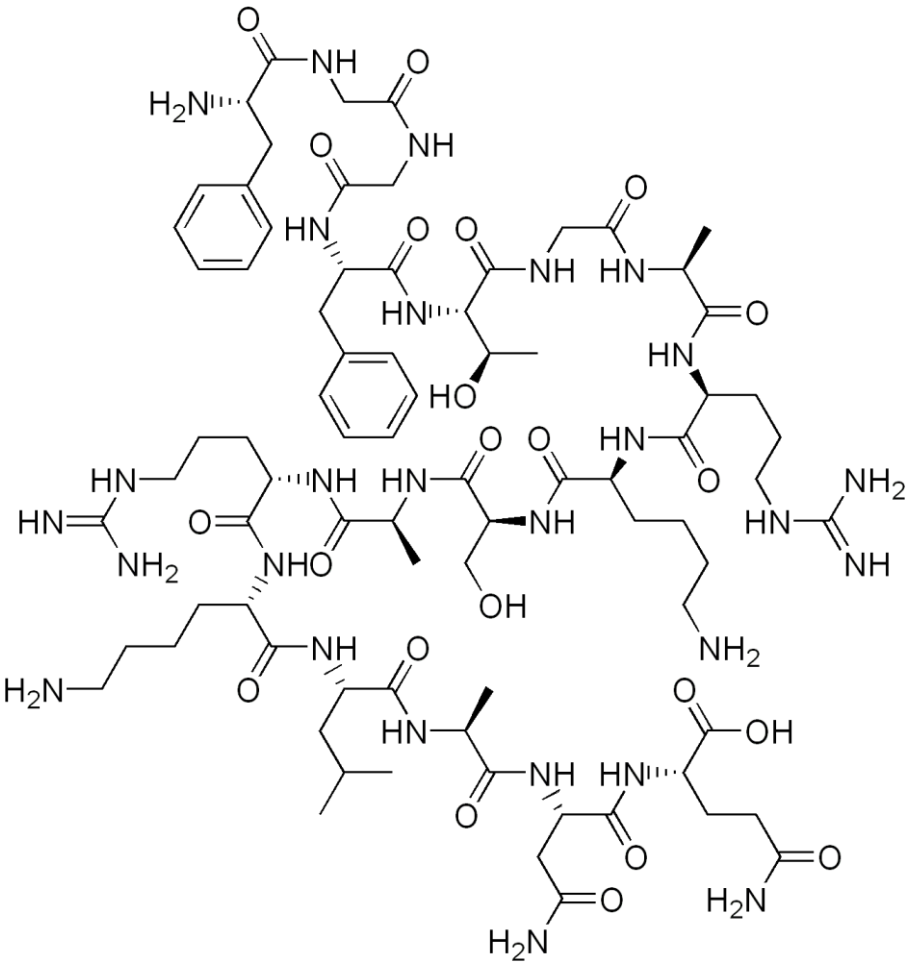
γ-Endorphin: Tyr-*Gly-Gly-Phe*-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu

Met-enkephalin: Tyr-*Gly-Gly-Phe*-Met

Leu-enkephalin: Tyr-*Gly-Gly-Phe*-Leu.

Figure 2: Structure of nociceptin and some of the closely related endogenous neuropeptides.

DOI:10.14753/SE.2016.1887



Molecular structure of nociceptin

1.1.2 Nociceptin receptor

Nociceptin (NC) receptor (NOP) in man consists of seven transmembrane regions with 370 amino acids [21]. The N-terminal of NC receptor polypeptide is a 44-amino acid unit with 3 adjoining sequences for glycosylation (Asn-X-Ser/Thr). In addition, protein kinase A and C can phosphorylate the polypeptide in the second and third intracellular loops, respectively. Numerous studies have described the structure of NC [11, 22, 23].

1.1.3 Distribution and localization of nociceptin and its receptor

NC and its receptors are widely distributed in the brain region like in thalamus, hippocampus, olfactory bulb, amygdala and cortical areas. [13, 21, 24–33]. NC receptors have been identified in other areas including the dorsal and ventral horns of the spinal cord [29, 34], periaqueductal gray matter of the midbrain and the nucleus raphe magnus.

NOP receptors co-localizes with *mu* opioid (MOP) receptors [26]. The localization and distribution pattern of NC-NOP system have revealed a role for NOP receptor in the processing of behavioural response to stress and anxiety, motor and balance control, aggression and autonomic control of physiological processes, reinforcement and reward, nociception and sexual behavior, [29, 34].

In addition, NC and its receptors have also been detected outside of the brain in peripheral organ systems such as spleen, vas deferens, intestine and immune system [27, 35, 36].

1.1.4 Physiological role of nociceptin

Nociceptin mediates the modulation of pain via stimulation of NOP in the brain and causes an increase in pain sensation, which suggests that NC may be implicated in the transmission of pain signals. In addition to the regulation of pain signals, NC has been implicated in a variety of physiological functions including, inhibition of locomotive activity [37], reversal of stressed-induced analgesia [37], reduction of stress responses [38, 39], induction of impairment in memory and learning [40, 41], release of neurotransmitters and hormones [42, 43], induction of diuresis and anti-natriuresis [44], neuronal differentiation [45], sexual and reproductive behaviour [46], itching, biting and licking [47], uterine contraction [48] feeding [49] anxiety [50–52], gastrointestinal motility [53]

induction of transient hypotension, diuresis-induced bradycardia [54–56], micturition [57] and antitussive effect in cough [58]. Additionally nociceptin may play a vital role in hypoxic-ischemic brain injury [59–62]. Many other functions regulated by NC include diuresis and sodium balance [44], regulation of temperature [63], vestibular function [64] modulation of inhibitory neural pathway that inhibit gastrointestinal movement, colonic and mucosal propulsive activity [65]. Nociceptin has been shown to suppress both excitatory [66] and inhibitory [67] synaptic transmission in the murine spinal cord. It also suppresses NMDA receptor-dependent long-term depression in the dentate gyrus of the hippocampus [68]. It also suppresses oxytocin, vasopressin and GnRH release [46] and inhibits tachykinin function [69]. Nociceptin activation of NOP is capable of modulating the activity of neurons in the suprachiasmatic nucleus [70] and lateral amygdale [71]. It has also been shown that nociceptin inhibits enkephalin release [72], mesolimbic dopamine transmission [73] and trigeminal neuronal response to excitatory amino acids [74]. In addition, nociceptin can inhibit endomorphin-1-induced analgesia [75]. In the periphery, N/OFQ inhibits nitric oxide release in the colon of murines [76]. OP_4/NOP receptor reduces the activation of adenylyl cyclase and Ca^{2+} channels while activating K^+ channels in a manner similar to opioids.

All of these reports show that nociceptin is indeed involved in a large number physiological functions (Table 1).

Table 1: Function of nociceptin in different biological systems

Biological systems	Biological functions
Brain Central nervous system Dorsal spinal cord Supra spinal	a. Anxiolytic-like actions b. Antidepressant-like actions c. Food intake (hyperphagia) d. Memory and learning process e. Modulation of locomotive activity f. Pain regulation g. Reward and addiction h. Thermoregulation (hypothermia) i. Regulates Addiction and anxiety-like behaviours
Cardiovascular system	a. Antiarrhythymic effect b. Decreases blood pressure and heart rate c. Produces hypotension and bradycardia d. Vasodilation (Cerebral) e. Vasodilation (Peripheral)
Endocrine system	a. Growth hormone secretion b. Prolactin secretion
Gastrointestinal tract	a. Inhibits motility (in mouse) b. Reduces intestinal motility and neurogenic contraction of stomach c. Stimulates motility (in rat)
Immune system	a. Analgesic effect b. Immune modulation c. Immunosuppression d. Increases vascular permeability under inflammation
Respiratory tract	a. Antitussive effect b. Inhibits bronchoconstriction
Renal function	a. Antinatriuresis b. Diuretic effect c. Inhibits micturition reflex
Reproductive system	a. Control of GnRH secretion b. Facilitates lordosis c. Facilitates erection d. Neuroendocrine control e. Regulating uterine contractility

Modified from Tariq et al. [77]

1.1.5 Nociceptin in pathological conditions

Plasma nociceptin concentration has been found to either increased or decreased in different pathological conditions **(Table 2).** *Albeit*, nociceptin is produced in neurons of the CNS and PNS [78] but there is growing evidence that it is also found in body fluids including the plasma, serum, and cerebrospinal fluid (CSF) [79]. The circulating nociceptin may be a good predictor and a biological marker for a variety of pathological conditions. However, few studies have been done on this topic. [80] noted that the CNS level of NC and prepronociceptin increased significantly in animal models of chronic constriction lesion and diabetic neuropathic pain.

Table 2: Circulating nociceptin (NC) in different pathological conditions

Pathological conditions	**Plasma level**
Acute and chronic pain	Increased serum NC level
Acute stroke	Increased plasma NC level
Angina	Increased plasma NC level
Atherosclerotic patients	Decreased plasma NC level
Bipolar disorders	Increased plasma NC level
Chronic ischemia of the limb	Decreased plasma NC level
Chronic Ischemic cardiovascular disease	Decreased Plasma NC level
Cluster headache	Decreased plasma NC level
Diabetic neuropathy	No significant change
Fibromyalgia syndrome	Decreased plasma NC level
Hepatocellular carcinoma	Increased plasma NC level
Major depressive disorder	Increased plasma NC level
Migraine without aura	Decreased plasma NC level
Primary biliary cirrhosis	Increased plasma NC level
Postpartum depression	Increased plasma NC level
Sepsis conditions	Increased plasma NC level
Transient ischemic attack	Increased plasma NC level
Wilson’s disease	Increased plasma NC level

Modified from Tariq et al. [77]

1.1.6 Nociceptin receptor (NOP) agonists and antagonists

In addition to the peptide ligands, many classes of non-peptide chemical NOP ligands have been synthetized and identified. They include piperidines, nortropanes, spiropiperidines, 4-amino-quinolines and quinazolines, and many others. A detailed study of NOP agonists and antagonists may be found in the following reviews [10, 77, 81, 82].

1.1.7 Effects of nociceptin in the pancreas and uterus

Pancreas

Tekes et al. [79] revealed that long-term diabetes mellitus does not modify the plasma or CSF levels of NC. The pancreas, which regulates a variety of imperative physiological activities, has also been studied in relation to nociceptin and NOP receptors. It is believed that prolonged and extended hyperglycaemia reduces pancreatic β-cell responsiveness to secretagogues [83]. A study was conducted by Linari [84] regarding the regulation of pancreatic exocrine secretion *in vitro* by NC revealed that the NC-NOP system plays an inhibitory role in the regulation of exocrine pancreatic secretion such as amylase. An experiment was conducted by Matsushita et al. [85] with intracerebroventricular infusion of NC to mice and observed an increased level of plasma insulin. In diabetic rats the role of NC in glucagon secretion still remains to be determined.

Uterus

Klukovits et al. [48] revealed the presence of PNOC in the uterus of rats using radioimmunoassay and radioligand-binding techniques. Deák et al. [86] reported that NC derived from PNOC relaxes uterine muscle in rat. Additionally, NC like any other classical opiates, seems to function as a neuromodulator of the endocrine system of the reproductive system. Bryant et al. [87] reported that NC increased prolactin release in both male and female rats. Using NC knock-out mice and their wild-type littermates as controls, they showed that NC is important in prolactin regulation during lactation. They also showed that offspring survival is decreased when NC is not expressed in post-partum dams. In addition, the role of NC was investigated during the post-partum period by Gu et al. [88]. They suggested that NC may play a role in the pathogenesis of postpartum depression. It is also

speculated that the secretion of corticotropin-releasing hormone (CRH) from the epithelium of the endometrium of uterine wall and other hormones secreted from the endometrium may be regulated by NC.

Another important physiological role of the uterine wall is its ability to contract, due to the presence of smooth muscle in the myometrium. Mollereau and Mouledous [89] reported the presence of NC receptors in smooth muscle cells, which is of course an indirect evidence of the presence of NC in smooth muscles. However, no morphological evidence is available for the presence of NC in the muscular layer of the uterus. Physiological studies have shown that NC relaxes the human uterus [86]. However,the exact nature of the role of NC in the uterus and on smooth muscle in general is far from clear, not to mention its pattern of tissue distribution.

1.1.8 Methods used to study localization and tissue distribution of nociceptin.

Several techniques have been employed to study different parameters including, light immunohistochemistry, and ultrastructural localization of NC in tissue and organ systems. Light and electron microscopy technoiques have been rarely employed to study the pattern of distribution of NC at both the tissue and cellular levels. However, further studies on the location of NC in tissues and cytoplasmic organelles are indeed necessary for understanding its potential clinical application. A basic, reproducible and widely applicable method is needed to achieve this target.

Apart from these research methods and techniques, antisense oligonucleotides targeting NOP receptors or PNOC genes, or applying antibodies against NC directly have been used. In some cases, the receptors or the peptide precursor genes have been deleted genetically in some animal models [9].

An extensive and robust investigation has been carried out in CNS of murine model to understand the distribution and pattern of NC and its receptors in the brain using *in situ* hybridization and immunohistochemical techniques [29, 90, 90–92]. There are some other detailed maps that have been compiled by Darland et al. [93] in which the expression of NOP receptors and PPNOC mRNAs where depicted in discrete areas of the rat brain.

Due to lack of appropriate techniques it has been difficult to determine the binding sites of NC in organ systems. In order to solve this problem, radioligand technique was used to determine the exact location of this peptide in the tissues. Unfortunately, the method has never been used again because it was not reproducible [77]. The basic cause of failure of this technique to localize the binding site of nociceptin was due to the choice of an inappropriate/insensitive technique or low tissue level of its receptors.

A summary of techniques and methods used to investigate the structure and distribution of NC is shown in Table 3.

Table 3: Methods used to study the structure and distribution of nociceptin in tissues and body systems.

Methods and Techniques	**Relevance/use**
Autoradiography	Expression of nociceptin receptors in tissues
DNA recombination technique (PCR etc.)	Detection of nociceptin in tissues
ELISA	Distribution in body tissues
Electrophysiological technique	Agonist and antagonists of NOP receptors
HPLC coupled to tandem mass spectrometry (LC-MS/MS)	Can be used for purification when combined with other techniques
Immunohistochemistry	Distribution in body tissues
In situ hybridization	Expression of the neuropeptide and its receptor
Light and electron microscopy	Immuno-gold and immuno-silver staining of nociceptin in intracellular organelles
Mass spectrometry	Structural elucidation, identification, characterization, metabolism study
Positron Emission Tomography	Imaging of distribution sites
Radioimmunoassay	Quantitative measurement of nociceptin
X-gal histochemistry	Detection of receptor-expressing cells

Modified from Tariq et al. [77]

1.1.9 Application of immunohistochemical, immunofluorescence and electron microscopy methods in the study of nociceptin

Literature search shows that electron microscopy (EM) is rarely used for the pattern of distribution and localization of NC in tissues even though it provides a solid and robust way of determining the cellular and intracellular localization. Electron microscopy comprises conventional transmission electron microscopy (TEM), immuno-EM whereas immunofluorescence microscopy is also a good tool for the morphological studies on nociceptin. In the electron microscopy, double-labelling immunocytochemistry is the most commonly used method for the detection of two or more peptides in cytoplasmic organelles [94].

1.2 Pancreas

1.2.1 Histology of pancreas

The pancreas has two functional components: exocrine part which produces pancreatic juice for digestion and endocrine component that produces insulin and other hormones such as glucagon, somatostatin and pancreatic polypeptide. The exocrine pancreas is formed mostly from acinar cells that secrete digestive enzymes and alkaline buffer to neutralize the acidic chyme formed by the stomach. These acini are surrounded by smaller ducts which drain into the central pancreatic duct. The gross morphology and histology of pancreas are shown in figures 3a and 3b.

The endocrine part of the pancreas is aggregated into small islands of cells called the islet of Langerhans. The islets are more abundant in the tail region of the pancreas. The capillaries of the islets are surrounded by layers of endocrine cells making direct contact with blood vessels. There are four types of cells in pancreatic islets which can be differentiated on the basis of their secretions. These are defined as α-cells which secrete glucagon, β-cells which secrete insulin, δ-cells which secrete somatostatin, and pancreatic polypeptide cells (PP-cells) which secrete pancreatic polypeptide [95].

1.2.2 Principal cell types of pancreatic islets

In normal pancreatic islets, the most numerous cell types are β-cells, which form about 70% of the total number of islet cells, and are located in the central portion and secrete insulin. The ultrastructure of pancreatric β-cell shows a homologous electron dense inner core, surrounded by an outer halo. The secretory granules are almost polyhedral in shape and a pale matrix and had a rounded or slightly oval nucleus with irregular contour and narrow perinuclear cisterns. The β-cells contain numerous secretory granules showing a mean diameter of 300 nm [94]. The α-cells constitute about 20% of the total cell population and are generally located peripherally in the islets. These cells are mostly polyhedral in shape and their nuclei are rounded with an undulating contour. The secretory granules of α-cells show homogenous, large rounded moderately dense cored with narrow lucent-halo or well fitted outer membrane. The α-cells secretory granules have a diameter of about 250 nm. The granules are more uniform in size and more densely packed in the cytoplasm than the granules of the β-cells [94] The δ-cells constitute about 5-10% of the total pancreatic endocrine tissue and are also located peripherally in the islets. The δ-cells secrete somatostatin, which is contained in secretory granules that are about 300-350 nm and contain material of low to medium electron density [94].

Polypeptide Pancreatic cells (PP-cells) are located in the peripheral part of the islet. The PP-cells are almost polyhedral in shape and show oval or rounded nuclei with slightly undulating contours. The cytoplasm is moderately granulated, showing immature secretory granules with variable electron dense rounded or oval homogeneous cores. Mature secretory granules having a core with high electron density are separated from the limiting membrane by a narrow electron-lucent halo. The diameter of the PP-secretory granules is 110 nm [94] .

1.2.3 Regulatory innervation of the pancreas

The pancreas receives regulatory stimuli via hormones in the blood and through the autonomic nervous system. These two inputs regulate the secretory activity of the pancreas. The pancreas is supplied with sympathetic adrenergic, parasympathetic cholinergic

neurotransmitters and neuropeptidergic nerves. These types of innervations are implicated in pancreatic secretory activities [96].

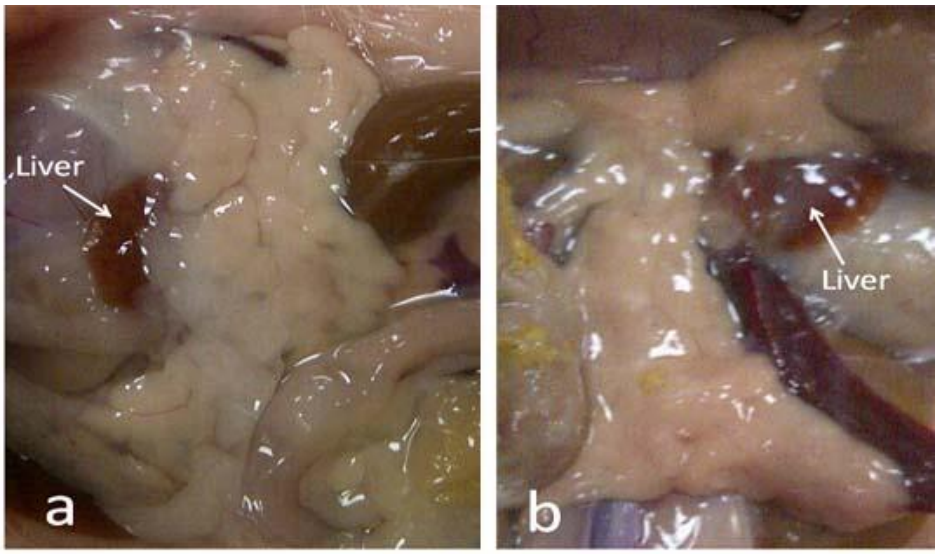


Figure 3a: Gross morphology of non-diabetic (a) and diabetic (b) rat pancreas.

Magnification: X2

DOI:10.14753/SE.2016.1887

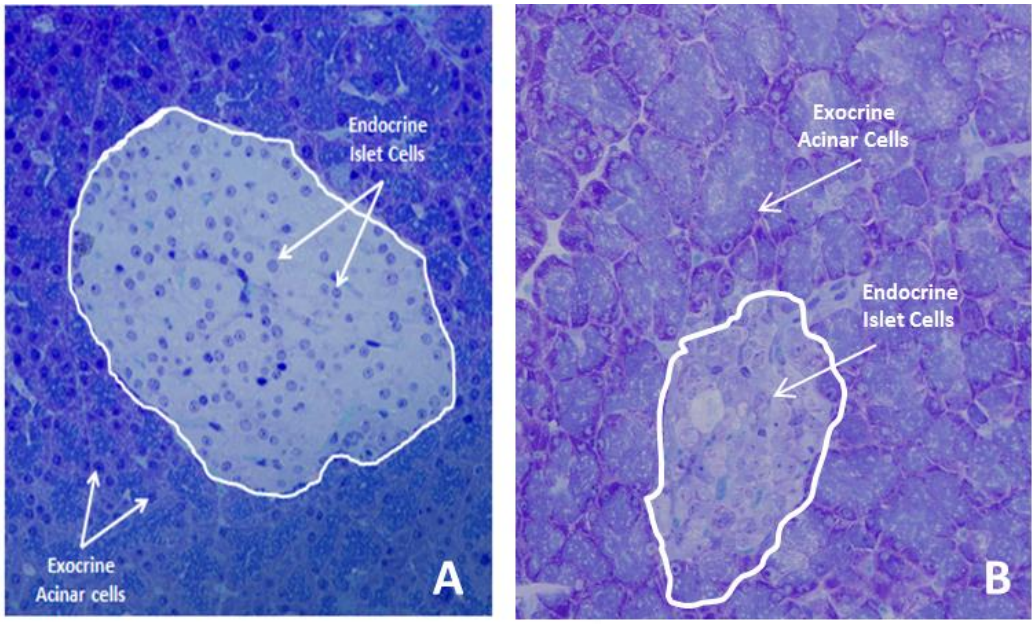


Figure 3b: Micrographs of the endocrine and exocrine pancreas of non-diabetic (A) and diabetic (B) rats. Magnification: x 400

DOI:10.14753/SE.2016.1887

1.3 Diabetes mellitus

Diabetes mellitus (DM) is a complex, chronic metabolic disease, has a heterogeneous group of symptoms and is characterized by disturbances of carbohydrate, fat and protein metabolism, in which the person has high blood glucose levels, either because the insulin production is inadequate, or because the body's cells do not respond properly to insulin, or both. Hyperglycaemia, or elevated blood glucose may lead to various long-term complications like neuropathy, retinopathy and cardiovascular problems [97, 98].

DM is also regarded as a chronic inflammatory disease caused by inflammatory cytokines involving the innate immunity. Modern research indicates that elements of the adaptive immune system may also contribute to this syndrome. Many reports show that inflammatory cytokines do indeed have major role in the pathogenesis of type 2 diabetes. The etiology of type 1 DM include autoimmune destruction of pancreatic β-cells [99, 100].

1.3.1 Types of diabetes

Type 1 diabetes mellitus (T1DM) results from the body's failure to produce enough insulin. T1DM is characterized by a massive loss or the necrosis of insulin-producing β-cells of the pancreatic islets of Langerhans, leading to insulin deficiency. This form was previously referred to as insulin-dependent diabetes mellitus (IDDM) or juvenile diabetes because β-cells are destroyed in childhood by autoimmune system. Autoimmune destruction of β-cells may be triggered by viruses or chemical toxins [101, 102]. T1DM accounts for about 10-15% of all cases of DM [97] .

Type 2 diabetes mellitus (T2DM) results from insulin resistance, a condition in which cells fail to use insulin properly. This form was previously referred to as non insulin-dependent diabetes mellitus (NIDDM) or adult onset diabetes. The defective responsiveness of body tissues to insulin is believed to involve the insulin receptor. However, the specific defects are not known. T2DM is the most common type of diabetes and it accounts for almost 85-90% of all DM cases [97, 103, 104].

Gestational diabetes: The third main form of DM is gestational diabetes which occurs when pregnant women without a previous diagnosis of diabetes develop high blood glucose level. It may precede the development of type 2 DM. Gestational diabetes mellitus (GDM) is the pathophysiological state of insulin resistance or reduced insulin secretion which is noticed for the first time during mid pregnancy and progresses through the third trimester [105].

1.3.2 Role of novel peptides in diabetes mellitus

Adipokines, cytokines and chemokines are the primary neuropeptides that play a role in insulin resistance in diabetes mellitus. Some of the neuropeptides and their role in diabetes are shown in **Table 4.**

Table 4: Novel peptides implicated in the development of type 2 diabetes mellitus

Neuropeptides	**Role in diabetes**	**Reference**
Acylation stimulating protein	Affects lipid and glucose metabolism. Leading to insulin resistance	[106]
Adipolin	Exerts beneficial actions on glucose metabolism	[107]
Adiponectin	Improves insulin sensitivity	[108]
Angiopoietin-like protein	Regulates ANGPTL3	[109]
Angiotensin	Serum level increases in oxidative stress, inflammation contributing to cell dysfunction in diabetes	[110]
Apelin	Decreases fat volume	[111]
Betatrophin	a) Disrupted triglyceride metabolism b) Controls pancreatic β cell proliferation	[112], [113]
IL-6	Increases circulating free fatty acid and decreases adiponectin concentration	[111]
Leptin	Regulates blood sugar via its control on appetite and fat storage	[114]

Lipasin	Regulates serum triglyceride levels	[115]
PAI-1	Modulate insulin signaling	[111]
RBP4	Involved in the pathogenesis of type 2 diabetes mellitus a)To link obesity with its comorbidities b)Insulin resistance, T2DM, and involved in certain components of the metabolic syndrome	[116]
Resistin	Causes resistance to insulin	[117]
RIFL	Involved in lipid metabolism	[118]
TGF-β	Increases in GDM	[119]
TNF-α	Contributes to the development of insulin resistance and diabetes	[111]
Vaspin	Insulin-sensitizing with cardioprotective and antiatherosclerotic in diabetes	[120]
Visfatin	Have insulin-mimetic actions	[111]

1.3.3 Nociceptin in diabetes mellitus

Peripheral neuropathy with significant neuropathic pain is a common complication of diabetes mellitus. It is widely accepted that nociceptin is involved in the pathogenesis of neuropathic pain caused by diabetes [121] and/or in pain regulation systems both at supraspinal and spinal levels. Intracerebroventricular injection of, nociceptin causes hyperalgesia or anti-opioid effects instead of analgesia. However, nociceptin produces allodynia or hyperalgesia when given intrathecally in low doses, but causes anti-nociceptive effects in high doses (Tekes [79, 80]. Liu et al. [80] found that , nociceptin concentrations are raised in the brain, spinal cord and serum of rats with diabetic neuropathy compared to control rats.

1.4 The uterus

The uterus of the rat consists of two horns, and is therefore referred to as a bicornuate uterus. At the tips of the two uterine horns are small lumpy glands called ovaries, which are connected to the horns of the uterus via tiny oviducts. The duplex structure of the uterus

DOI:10.14753/SE.2016.1887

enables the rat to have multiple embryos [122]. The gross morphology and histology of uterus are shown in figures 4 and 5.

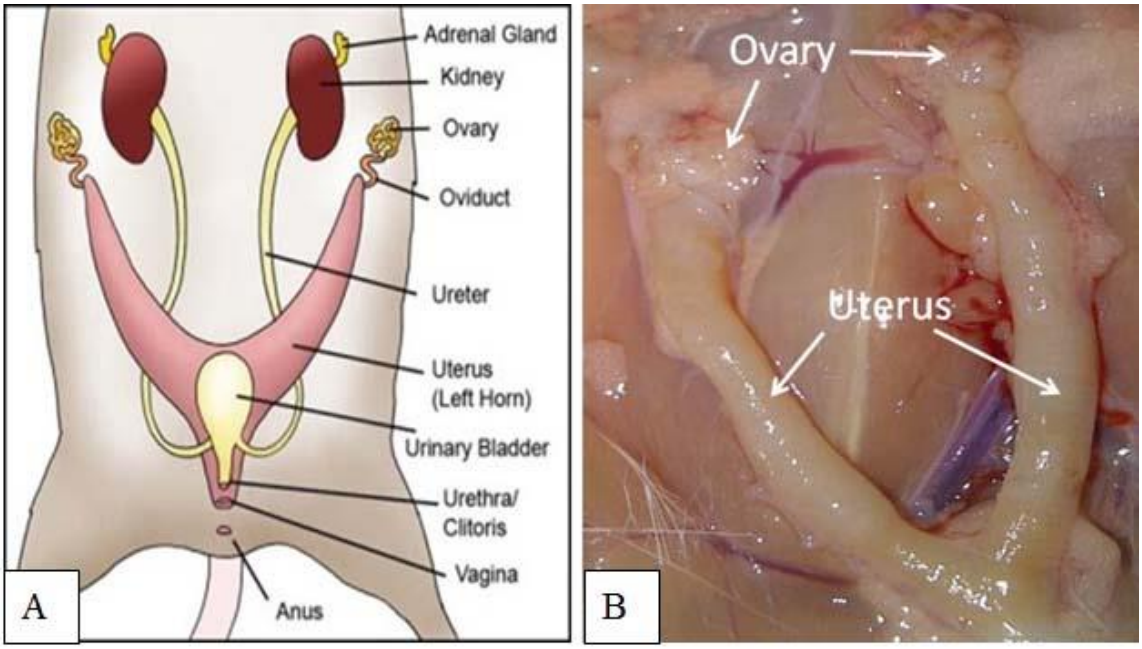


Figure 4: Gross morphology of the rat uterus (Fig 4A showing the reproductive system of the female rat which has been modified from [123] and in Fig 4B (arrows) shows the ovary and uterus, respectively. Magnification of 4B=X2

1.4.1. Histology of the rat uterus

1.4.2 The uterine wall

The uterine wall is composed of three layers of tissue. The three layers, from the innermost to the outermost, are as follows endometrium, myometrium and perimetrium [124].

Endometrium: The lining of the uterine cavity is called the endometrium. It consists of functional and basal layers. The functional layer can be further divided into compact and spongious compartments. Blood vessels that supply the functional layer arise from the basal layer. In all placental mammals, the endometrium is shed off periodically or reabsorbed if no pregnancy occurs. Thickness of endometrium of rat varies greatly and ranges from 0.5 mm to 5 mm and depends upon the reproductive cyclic changes [125].

The glandular and vascular tissues of the endometrium support the physiological demands of the growing foetus. Large numbers of uterine glands open onto the endometrial surface and extend deep into the lamina propria, almost reaching the myometrium.

Myometrium: The myometrium is the thickest layer of the uterine wall. The myometrium mostly consists of smooth muscle cells, connective tissue and contains larger blood vessels. Moreover, it is composed of three indistinctly defined layers of smooth muscle. The middle muscular layer contains numerous large blood and lymphatic vessels. The inner and outer layers of muscles are predominantly oriented parallel to the long axis of the uterus. The myometrium provides much of the contractile force needed to move a large foetus out of the uterus [126].

Perimetrium: The perimetrium is a loose connective tissue around the uterus. This layer is continuous with the lining of the pelvis. The perimetrium surrounds the posterior uterine surface and a significant part of the anterior part. The lower part of the anterior surface is lined by connective tissue [127]

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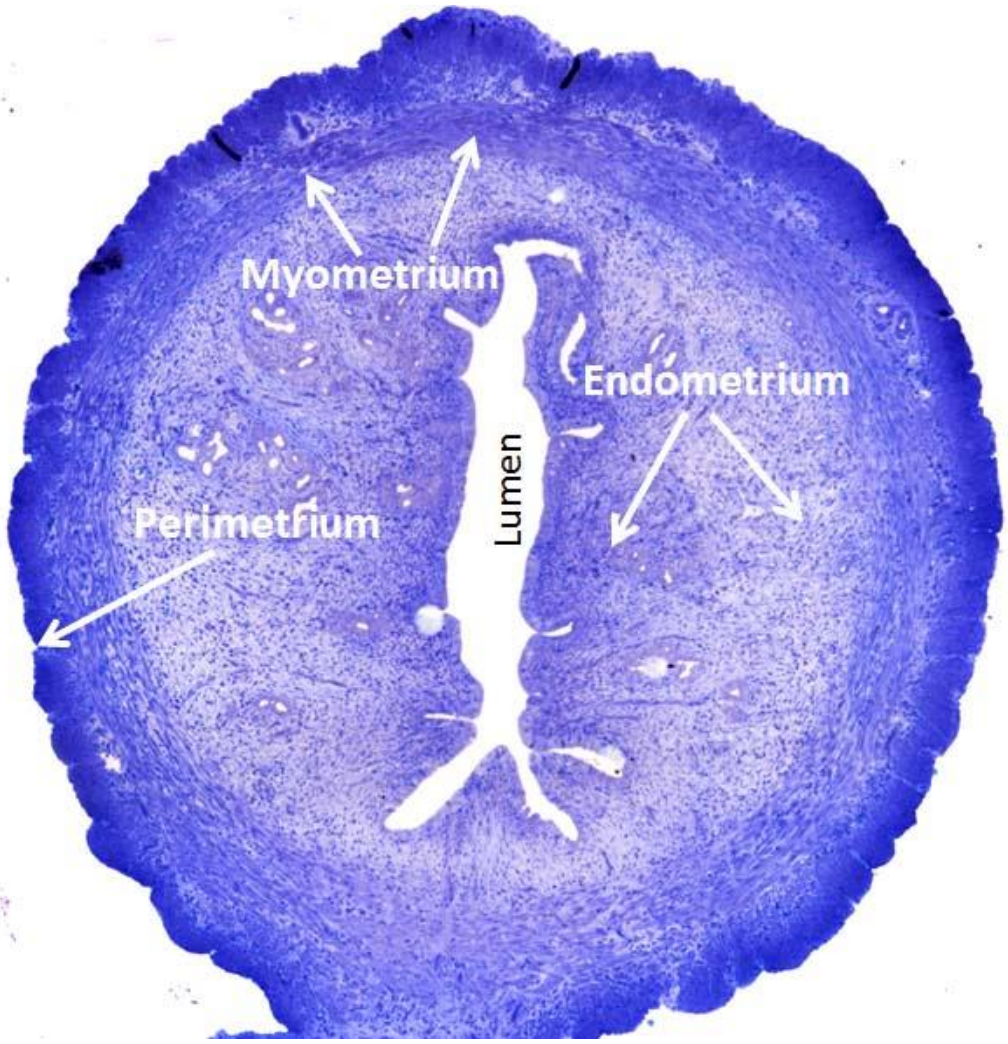

Figure 5: General histology of the rat uterus showing, perimetrium, myometrium, endometrium (arrows) and lumen. Magnification: X40

DOI:10.14753/SE.2016.1887

2. Hypotheses, Aims and Objectives

Hypotheses

Nociceptin has been implicated in the physiology of pain, including those associated with labour. Nociceptin has also been shown to play a role in endocrine secretion. The aim of the study is to test two hypotheses: Nociceptin is present in endocrine glands e.g. endocrine pancreas. This will allow the nociceptin to play a role in endocrine function.

1. Nociceptin, which has also been localized to smooth muscle, is present in the uterus. The presence of nociceptin will enable it to participate in uterine pain especially during labour.
2. The tissue content of nociceptin will change after the onset of diabetes mellitus.

Aims of the study

The aims of the study were to:

1. Determine the pattern of distribution of nociceptin in the pancreatic islets cells of rats. Determine the localization of nociception in the uterus tissue of rats.
2. Determine the hypothetic changes of the nociceptin level in the uterus of murine model of streptozotocin-induced diabetes

Objectives of the study

1. Investigate the distribution of nociceptin in the endocrine pancreas of non-diabetic and diabetic rats using immunohistochemical, immunofluorescence, Western blot and immunoelectron microscopy methods.
2. Examine morphology of the uterus after the onset of diabetes.
3. Examine the ultrastructure of the endometrium and myometrium of non-diabetic and diabetic rats using conventional electron microscopy.
4. Investigate the distribution of nociceptin in the uterus of non-diabetic and diabetic rats using immunofluorescence, Western blot and immunoelectron microscopy methods.

DOI:10.14753/SE.2016.1887

3. Materials and Methods

3.1 Experimental animals

Twelve adult male or female Wistar rats, weighing 225-250 g, were divided randomly into non-diabetic control group (n = 6) and streptozotocin (STZ)-induced diabetic group (n = 6). Wistar rats were procured from Harlan Laboratories (Harlan Laboratories, Oxon, England) and bred from the original stock in the Animal Facility of the College of Medicine, UAE University. The rats were housed in plastic cages (six rats/cage) in climate-controlled facilities at 23 ± 1^{o}C and 50 ± 4% humidity. Day and night cycle was maintained at 12 h/12 h. Standard animal chow (Emirates Feed Factory, Abu Dhabi, UAE), and tap water were provided *ad libitum*. The study was performed with the approval of the CMHS Animal Research Ethics Committee (Approval number: A02/11). During the entire study, the Helsinki guiding principles for the care of and use of laboratory animals were been observed.

3.2 Induction of experimental diabetes mellitus

Diabetes mellitus (DM) was induced either in male or female rats by a single intraperitoneal injection (ip) of streptozotocin, (STZ) (Sigma Chemical Co., St. Louis, MO, USA) at a dose of 60 mg/kg body weight [128]. The STZ was freshly dissolved in citrate buffer (0.5 M, pH 4.5) (see appendix). Following seven days after STZ injection, DM was confirmed by checking blood glucose with test strips using One Touch Ultra 2 glucometer (Life scan Inc., Milpitas, CA, USA). Drops of blood from the tail end of each rat were used for this purpose. The rats were considered diabetic if the random blood glucose levels were ≥ 280 mg/dl. The animals were processed for morphological studies two weeks after the onset of DM. Body weight and blood glucose levels were noted before and at the end of the experiment.

3.3 Experimental design

Following the diagnosis of DM, both age-matched healthy non-diabetic controls and STZ-induced diabetic male or female virgin rats were divided into two groups each containing six rats. Female rats were selected on the basis of their oestrous cycle as well [129]. Rats with oestrous cycle on days four were selected for the study. The selection was based on

the visual nature of the external genitalia including swollen, moist, and pink vaginal opening with wrinkles and or striations in the posterior and anterior borders.

Groups of experimental animals

- Group 1: Non-diabetic healthy controls
- Group 2: Diabetic rats

3.4 Body weight

The body and organ (either pancreas or uterus) weights of non-diabetic and diabetic rats were recorded using a 9001 Scale (Sartorius, Hertfordshire, UK) and expressed as mean ± standard error of the mean (SEM) of the body or organ weights.

3.5 Glucose measurement

The blood glucose level was measured for each individual rat of both groups. The blood samples were drawn from the tail end of the rat for blood glucose measurement and expressed as mean ± standard error of the mean (SD).

3.6 Glucose tolerance test on non-diabetic and diabetic rats

Non-diabetic and STZ-induced diabetic rats were subjected to intraperitoneal (i.p) glucose tolerance test, after an overnight fasting for 12 hours. Each rat was given an i.p. glucose load of 2 g/kg body weight according to the method of Kim et al. [130]. The blood glucose measurements were made at fasting zero time (before glucose load), 30, 60, and 120 minutes after the glucose load.

3.7 Tissue collection

Two weeks after the onset of DM, all of the rats from each group were killed humanely under general anesthesia by diethyl ether. A mid-line abdominal incision was made and either the pancreas or uterus was rapidly removed. Representative tissue fragments were taken from the body of the pancreas and uterus and used for immunohistochemical, immunofluorescence, electron microscopy and molecular biology studies.

3.8 Light microscopy of pancreas and uterus

Pancreas and uterine tissues from non-diabetic and diabetic female Wistar rats (non-diabetic control n=6) and (diabetic n=6) were dissected out and washed in phosphate buffered saline (PBS) at pH 7.2, blot dried, cut into small pieces and fixed rapidly for 24 hours in freshly prepared Zamboni's fixative [131]. After washing with running tap water, the samples of pancreatic tissues were dehydrated in ascending series of concentrations of ethanol and cleared in xylene and then embedded in paraffin wax for immunofluorescence and immunohistochemical analysis according to standard procedures [132]. The paraffin blocks were trimmed with razor blade and then sections of 5-6-μm thickness were cut with rotary microtome and were placed on slides and stored until further processing.

3.9 Immunohistochemical studies of pancreas

Deparaffinized and rehydrated sections were processed for immunohistochemistry using the avidin-biotin complex (ABC) method [133].

Briefly, the sections were incubated in 0.3% H_2O_2 solution in methanol for 45 min to block the activity of endogenous peroxidase. Then the sections were rehydrated in descending, gradeed ethanol (100% to 50%) and then transferred into phosphate buffered saline (PBS) three times (5 minutes each). After washing with PBS the tissue sections on the slides were encircled with Dako pen (Dako Cytomation, Glostrup, Denmark) to stop the draining of the solutions from the sections.

The sections were later incubated with blocking buffer for 30 min to start the staining procedure. After draining off the blocking buffer, the sections were allowed to incubate overnight in primary antibodies (anti-rabbit nociceptin antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:100 at 4°C. On the next day the slides were removed from fridge and kept at room temperature for one hour and were later washed 3X in PBS (5 minutes each).

Then the slides were incubated with pre-diluted biotinylated anti-rabbit IgG for 1 hour and again the sections were washed with PBS 3 times (5 minutes each).

Then sections were later incubated in streptavidin peroxidase conjugate at a dilution of 1:1000 for one hour and then washed in PBS 3 times (5 minutes each).

The peroxidase activity was observed by incubating the sections in 3,3-diaminobenzidine tetrahydrochloride (DAB) containing 0.03% hydrogen peroxide in PBS for 5 minutes.

Later the slides were then washed under running water for 5 min and then counterstained with haematoxylin for one min. They were later differentiated in acidic ethanol and washed for 2 min with running tap water, then again dehydrated in ascending grades of ethanol for 3 minutes each (50%, 70% & 95% ethanol) and then in 100% ethanol with two changes for 5 min each.

Subsequently the sections were cleared in xylene and mounted on glass slides in Cytoseal 60 (Stephens Scientific, Riverdale, New Jersey, USA). The sections were examined with Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany) and the images of the immunopositive cells were taken.

3.10 Double-labelling immunofluorescence studies of the pancreas

In order to determine whether NC colocalizes with insulin in non-diabetic and diabetic pancreatic tissues, sections were incubated with antibodies against NC and insulin before being immunolabelled with either tetramethylrhodamine isothiocyanate (TRITC; red) or fluorescein isothiocyanate (FITC; Green) according to a previously described method [133].

Deparffinized sections were washed in PBS and circled with a Dako pen to prevent solutions draining away from the tissue section. The sections were incubated first with blocking reagent for 30 min.

Thereafter, the blocking reagent was drained off and the sections were later incubated with the primary antibody (anti rabbit nociceptin polyclonal antibody; 1:100) for 24 hours at 4^{o}C. On the following day, the sections were brought to room temperature for one hour and after washing with PBS, the sections were labelled with secondary antibody (anti rabbit TRITC; 1:100) for one hour at room temperature. The same pancreatic tissue sections were

later incubated with the second primary antibody (anti mouse insulin antibody) overnight at 4^{o}C and then labeled with the second secondary antibody (anti mouse FITC; 1:100) (Jackson Laboratory, West Grove, Pennsylvania, USA) for one hour. After washing with PBS three times each, the tissue sections were held in Immunomount® (Shandon, Pittsburgh, PA, USA). Sites of immunoreaction were detected and photographed with Zeiss confocal microscope (Carl Zeiss, LSM 510, confocal microscope Jena, Germany).

3.11 Immunofluorescence microscopy of the uterus

In order to determine the localization of NC in the endometrium or myometrium, deparaffinized sections were incubated with antibodies against NC. Immunolabelling with fluorescein isothiacyanate (FITC) was employed according to a previously described method [133]. Briefly, sections of the uterus of non-diabetic control and diabetic rats were treated with a blocking agent for 30 min at room temperature after rinsing in PBS. The sections were then incubated with anti rabbit nociceptin polyclonal antibody (1:100) for 24 hours at 4^{o}C. Sites of immunoreaction were detected with anti-rabbit FITC (1:100). The sections were held in Immunomount® mounting medium (Shandon, Pittsburgh, PA, USA), examined, and images were taken with a Nikon Confocal Microscope Eclipse 80i, Japan.

3.12 Tissue processing for conventional electron microscopy

Pancreatic and uterine tissues were dissected out from each group of rats (non-diabetic and diabetic) washed in 0.1M phosphate buffer (pH 7.2) and then immersed immediately in Karnovsky's fixative at pH 7.2 [134] for 24 hours at 4^{o}C. After rinsing with phosphate buffer the tissue samples were post fixed with 1% osmium tetroxide for one hour. After washing with distilled water, the samples were dehydrated in ascending series of graded ethanol from 30% to 95% and 100 % and then finally in propylene oxide. Then tissue were infiltrated and embedded in Agar100 epoxy resin and polymerized at 65^{o}C for 24 hours. Blocks were trimmed and semithin and ultrathin sections were cut with Reichert Ultracuts, ultramicrotome. Semithin sections (130 nm) on glass slides were stained with 1% aqueous toluidine blue on electro thermal slide drying bench at 55^{o}C and ultrathin sections of golden colour (95 nm) on 200 mesh copper grids then were contrasted with uranyl acetate [135] followed by lead citrate [136]. Then grids were examined and photographed at different

magnifications with Philips CM10 transmission electron microscope (Eindhoven, Netherlands).

3.13 Double-labelling immunoelectron microscopy of the pancreas

Fixation of tissue samples

Immunoelectron microscopic studies were carried out by using a previously described method [137]. Pancreatic tissue fragments of six, non-diabetic (n =6) and six diabetic (n = 6) male Wistar rats for each group were used for the immunoelectron microscopy study. Rat pancreata were quickly excised and cut into pieces (4 x 4 mm^3) and fixed in freshly prepared 4% p-formaldehyde + 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.2 [138] fixative for 24 hours at 4^0C. Dehydration and embedding of the samples into LR white resin (London Resin, Agar Scientific, UK) was performed according to [139] techniques with some modifications.

Embedding of tissue samples in LR White resin and sectioning

After washing in the fixation buffer, the tissues were dehydrated through a graded series of ethanols from 70%-95%. The dehydrated samples were infiltrated with three mixtures of ethanol and LR white resin (2:1, 1:1, 1:2) staying one hour at each stage. Afterwards, they were transferred into pure LR white resin for 24 hours at 4^0C. The samples were then placed into fresh embedding medium for 2 hours at room temperature. Then the tissues were later transferred into Agar gelatin capsules of 0 sizes with labels and filled with fresh LR white resin and tightly capped. Polymerization was achieved by irradiation with ultraviolet light (360-365nm) for 24 hours in a TAAB UV chamber at room temperature. Capsules were removed and blocks were trimmed. Semithin and ultrathin sections were cut with Reichert Ultracuts (Leica Grermany) ultramicrotome. Semithin sections were placed on a drop of water on glass slides and were dried and stained with 1% toluidine blue on a hotplate. Ultrathin sections (golden colour, 95 nm) were cut and collected and mounted onto carbon formvar-coated 200 mesh nickel grids.

Postembedding double immunolabelling of ultrathin sections

Nickel grids were then jet-washed with deionized water thoroughly to remove aldehydes from the sections, then the grids were placed in aqueous 10 % H_2O_2 for 10 minutes. After washing in deionized water the sections were immersed in 0.5 M NH_4Cl in 0.01 M PBS (pH 7.2) for 20 min to reduce the staining background. After washing with PBS buffer (pH 7.2) containing, 1% BSA and 0.1% Tween-20 for 5 min, they were then blocked in 20% NGS diluted in washing buffer for 10 min. The grids were later incubated overnight at 4°C with nociceptin at 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and washed in PBS and blocking buffer. They were later incubated with goat anti-rabbit IgG conjugated to 10 nm gold particles at a dilution of 1:20 for two hours at room temperature.

After washing in PBS, the grids were incubated in the second primary antibody (antibodies against insulin, 1:100; Dako, Copenhagen, Denmark) for 24 hours at 4^oC. The sections were brought to room temperature for one hour and incubated again in a solution of goat anti-mouse IgG conjugated to 5 nm gold particles at a concentration of 1:20 for 2 hours at room temperature.

After washing with PBS buffer, the sections were fixed in 2.5% aqueous glutaraldehyde and were washed with deionized water then blot dried. The grids were contrasted with 2% uranyle acetate and lead citrate, for 15 and 7 minutes, respectively. After washing the grids with deionized water and dried on a filter paper, they were examined with Philips CM10, transmission electron microscpe (Eindhoven, Netherlands).

3.14 Immunoelectron microscopy study of uterus

Immunoelectron microscopy study was performed according to the methods described by [94, 140]. Briefly, uterine tissue fragments of non-diabetic control and diabetic Wistar rats were dehydrated in ascending concentrations of ethanol (50% to 70%) and transferred to zero-sized Agar gelatin capsules then embedded in LR white resin as described in section 3.13.

Postembedding single immunolabelling of ultrathin sections

Ultrathin sections were cut and stained as described in section 3.13. Briefly, the uterine tissue sections were incubated overnight with antibodies against nociceptin (1:100; Santa Cruz) at 4°C in humid chamber. The next day the grids were brought to room temperature

for one hour then washed and incubated with secondary anti-rabbit IgG conjugated to 10 nm gold particles, at a concentration of 1:20. After washing with PBS the sections were fixed in 2.5% aqueous glutaraldehyde solution, washed with deionized water and then blot dried. The grids were later contrasted with 2% uranyl acetate and lead citrate, for 15 and 7 minutes, respectively. After washing the grids with deionized water, they were dried on a filter paper and stored in petridish. The grids were examined and images were taken with Philips CM10, transmission electron microscope (Eindhoven, Netherlands)

3.15 Morphometry

Six images from each group were randomly selected for quantification. The procedure was carried out on immunohistochemical, immunofluorescent and immunogold electron microscopy images. For the immunohistochemical images, the results were shown as percentages of labeled immunoreactive cell related to the total number of cells (± SEM). In immunofluorescence images, cells containing both nociceptin and insulin were counted.

In transmission electron microscopy images, quantification of nociceptin and insulin peptides were based on secretory granules of β-cells that contain both nociceptin- and insulin-labelled gold particles, 10 nm for nociceptin and 5 nm for insulin. The number of gold particles present on secretory granules was counted and reported to the total number of labelled granules, thus providing a mean density of labeling in each secretory granule.

3.16 Western blotting of nociceptin in tissues

A. Preparation of pancreas and uterine tissues lysates

1. The uteri and the pancreata were retrieved from the animals and kept on ice to prevent tissue lysis.
2. After homogenization, the tissue samples were placed in Eppendorf tubes and stored at -20°C immediately. To a 5 mg piece of tissue, 300 µl lysis buffer (RIPA buffer) was rapidly added before homogenization using electric homogenizer. The blade was rinsed twice with another 2 x 300 µl lysis buffer, then maintained at constant agitation on an orbital shaker for 2 hours at 4°C.

3. The homogenate was centrifuged at 10,000 rpm at 4°C in a microcentrifuge for 10 minutes. The supernatants were transfered to a fresh tube kept on ice. The pellet was then discarded.

B. Protein Quantification

1. A small volume (5µl) of supernatant was taken from the total protein extract to perform quantitation using BSA as standard (Bio-Rad Laboratories, Hercules, CA, USA, Cat no. 500-0001). The protein concentration was determined for tissue lysate using Bio-Rad protein Assay kit, (Cat no. 500-0001).
2. To the remaining volume of tissue lysate, an equal volume of 2X Laemmli sample buffer (Loading buffer) was added.
3. Tissue lysate was boiled in sample buffer at 95°C for 10 minutes and aliquot to reduce and denature the protein and stored at -20°C.
4. Prior to electrophoresis, the tubes containing cell lysate were defrosted at 37°C on ice and centrifuged at 16,000 x g in a microcentrifuge for 5 minutes at 4^{o}C.

C. Sample protein loading and separation using gel electrophoresis

The quantity of protein samples loaded into the wells of 10 % SDS-PAGE gel was 40µg along with prestained, molecular weight ladder. 10X diluted running gel buffer was diluted with distilled water up to 1X and filled the tank. The gel was run for 2-3 hours at 90 Volts.

D. Transfer of protein samples from the gel to the membrane

The gel was placed in transfer buffer for 10 minutes and the stack was transferred as follows:

1. The gel was placed on the cathode side and the blot on the anode. The cassette was gently placed in the transfer tank containing transfer buffer and placed on ice block in the tank. The protein was transferred from the gel to nitrocellulose

membrane for 2 hours at 4°C in the dark at constant current of 90 volts. The membrane is now ready for antibody staining.

E. Ponceau Staining

The membrane was rinsed in water and incubated in 100% Ponaceau S stain until bands appear to check the transfer quality. Wash off the Ponaceau S stain was washed off with three washes in tris-buffered saline tween-20 (TBST) until proper cleaning.

F. Staining of uterine and pancreatic samples with antibody

The gel membrane was blocked with neutral protein for one hour at 21^{o}C, shaked and mixed continuosly with 5% non-fat milk powder in Tris Buffered Saline with Tween® 20 (TBST).

The membrane was incubated with a primary antibody (anti-nociceptin antibody, ab10277) at a concentration of 1:500 in 5% blocking solution overnight at 4°C.

1. GAPDH (14C10) Rabbit mAb #2118 was used as loading control.
2. Then membrane was washed 3X in TBST for 5 minutes each and then incubated with secondary antibody (goat anti-rabbit IgG-HRP Conjugate #170-6515, Biorad) at 1:5000 dilution in blocking buffer containg 5% milk in TBST at room temperature for one hour.
3. The membrane washed 3X in TBST for 5 minutes each, then rinsed in TBS.
4. The signal was developed by using, Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific Inc.81 Wyman Street Waltham, MA USA 02451) according to manufacturer's protocol.
5. The images were captured with Typhoon FLA 9500. (GE Healthcare Bio-Sciences AB Björkgatan 30 751 84 Uppsala Sweden)

DOI:10.14753/SE.2016.1887

3.17 Statistical analysis

Statistical analysis was done by using SPSS software 21.0. The data obtained were calculated as means ± SEM. Comparisons between data were done and non-parametric Mann-Whitney U test was used to determine the statistical significance. P values of less than 0.05 were taken as significant.

DOI:10.14753/SE.2016.1887

4. Results

4.1 Pancreas

4.1.1 Body and organ weight ratios

The average body weight of the non-diabetic male rats was 218±12 g. Pancreas to body weight ratio for non-diabetic rats was 0.0020±0.0004. The average body weight of diabetic rats was 185±10 g and pancreas to body weight ratio was 0.0040±0.0007. Statistical analysis using Mann Whitney test reveals no significance.

4.1.2 Glucose measurement

The random blood glucose level was measured for each individual rat of both groups. The average blood glucose level for non-diabetic controls was 121 ± 12 mg/dL. The average blood glucose level for diabetic rats was 579 ± 10 mg/dL. There was a marked ($p < 0.05$) difference between the glucose level in non-diabetic controls versus diabetic rats.

4.1.3 Glucose tolerance test in male rats

Figure 6 depicts changes in the blood glucose concentration during intraperitoneal glucose tolerance test. The glucose load of 2g/kg body weight intraperitoneally generated different profiles in control and STZ-diabetic group. The changes in glucose levels in non-diabetic rats differed from those in the diabetic group. Basal plasma glucose was significantly higher in the diabetic rats and the i.p. glucose load increased glucose levels, in both groups but the level of blood glucose remained significantly ($p < 0.05$) higher in diabetic rats even 120 minutes after glucose challenge.

DOI:10.14753/SE.2016.1887

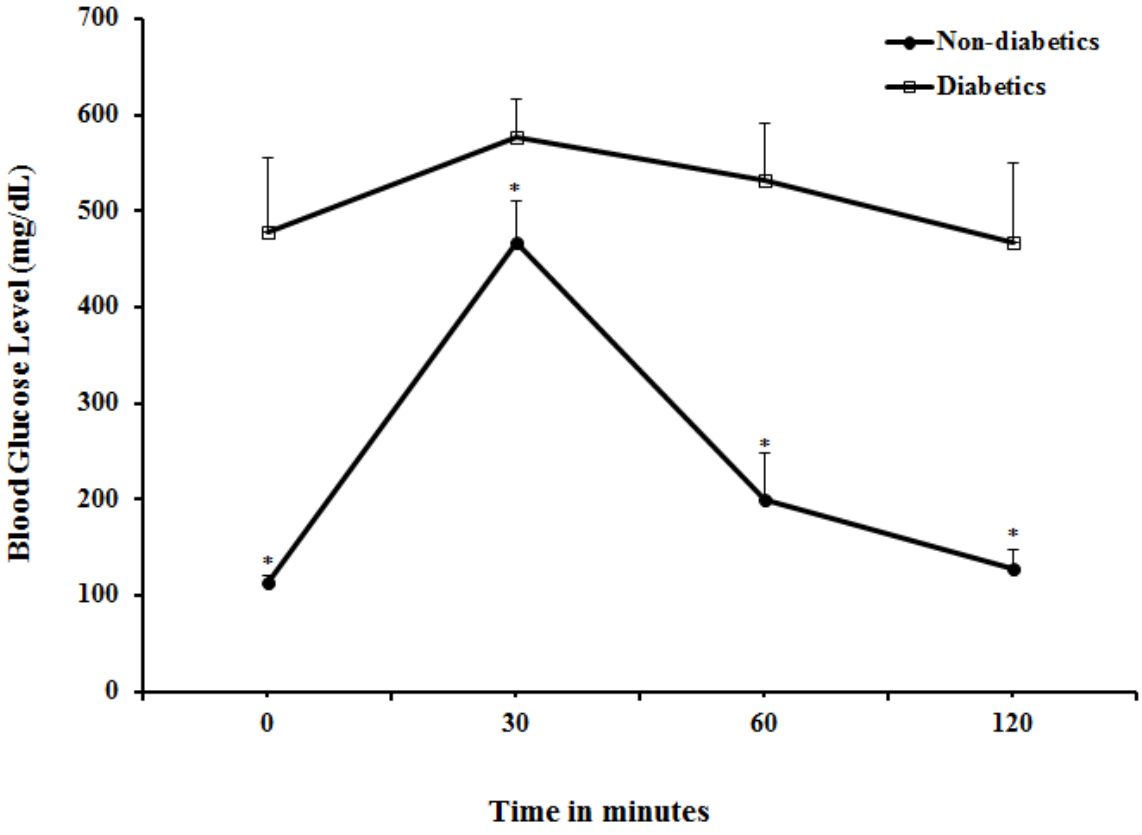

Figure 6: Glucose tolerance test in male Wistar rats after i.p. load of 2g/kg of glucose.

*$p < 0.001$ (non-diabetic versus diabetic**)**

DOI:10.14753/SE.2016.1887

4.1.4 Light microscopy for pancreas

Immunohistochemistry by avidin-biotin-complex and double labelled immunofluorescence methods were employed to determine the presence of NC in pancreatic tissues of non-diabetic control and diabetic rats. The results are summarized below.

4.1.5 Immunohistochemistry studies (Avidin Biotin Complex method)

Figure 7 shows nociceptin immunoreactive cells in the central and peripheral portions of the islet of Langerhans of non-diabetic control rats. According to Figure (7a), the number of nociceptin-positive cells in the pancreatic islet of non-diabetic control rats appears to be higher compared to those seen in the islets of diabetic rats (Figure 7b). The sizes of the islets were found to be reduced in diabetic condition (highlighted with arrows).

DOI:10.14753/SE.2016.1887

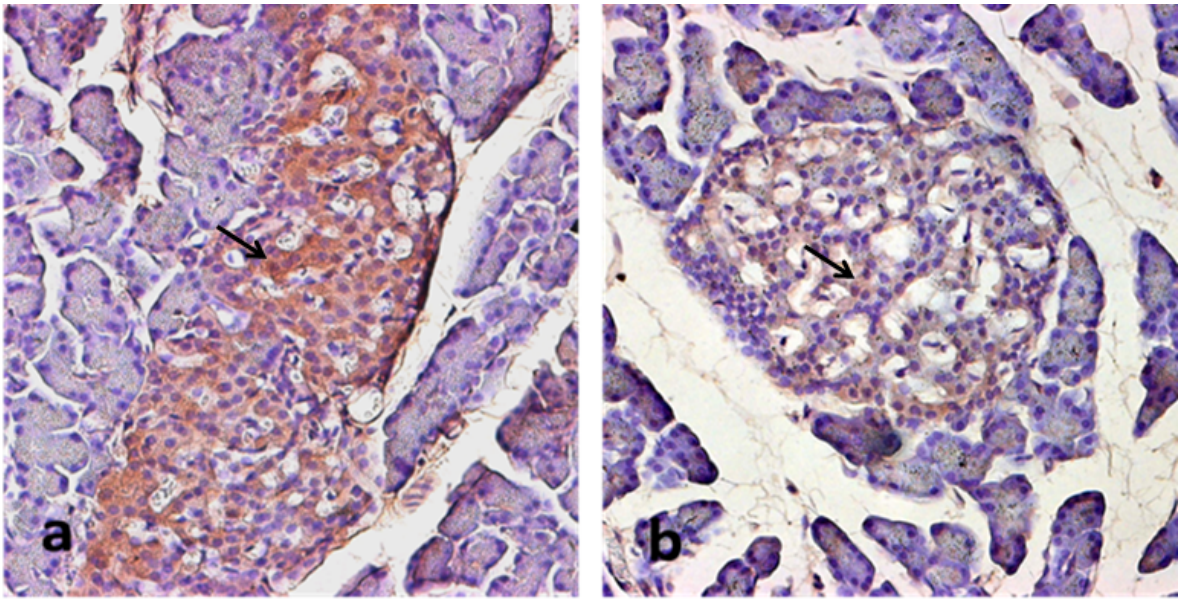

Figure 7: Light microscopic images of nociceptin-immunopositive cells (arrows) in the endocrine pancreas of non-diabetic (7a) and diabetic (7b) rats. Note that the expression of nociceptin is stronger in the islets of non-diabetic control compared to that of diabetic rats. Magnification: X400

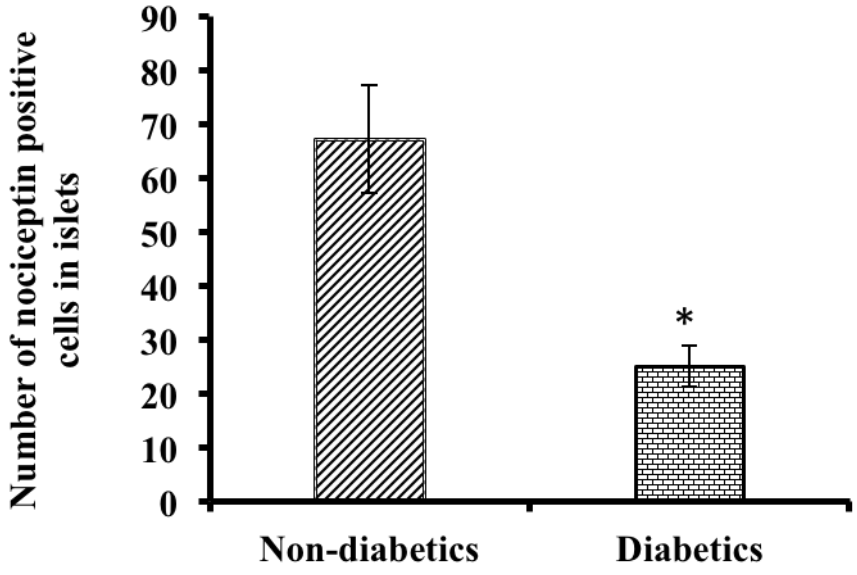

Figure 8: Percentage distribution of nociceptin-immunoreactive cells in the pancreas of non-diabetic and diabetic rats.Note that the number of nociceptin-positive cells is significantly ($p < 0.05$) lower in diabetic rats - controls. n=6

4.1.6 Double labelling immunofluorescence study

Immunofluorescence technique further corroborates our findings with the ABC method as reported in earlier section (Figure. 7a and b). The result of the immunofluorescence study is shown in Figure 9 which illustrates the co-localization of nociceptin with insulin. Nociceptin-positive cells are shown in red while that of insulin by green fluorescence. Yellow colour specifies cells that contain both nociceptin and insulin.

A representative section of non-diabetic rat islets is shown in Figure 9a and diabetic in Figure 9b. The pancreatic islets of non-diabetic control rat pancreas contain significantly ($p \leq 0.05$) greater number of NC- and insulin-positive cells compared to diabetic group (Fig. 9). The figures show that insulin and nociceptin immunoreactivity can be observed in β-cells of pancreatic islet. In figure 9c morpohmetric analysis also shows the number of cells containing either insulin, nociceptin or insulin+nociceptins significantly lower (*****$p< 0.0000003$) compared to non diabetic rat pancreas.

DOI:10.14753/SE.2016.1887

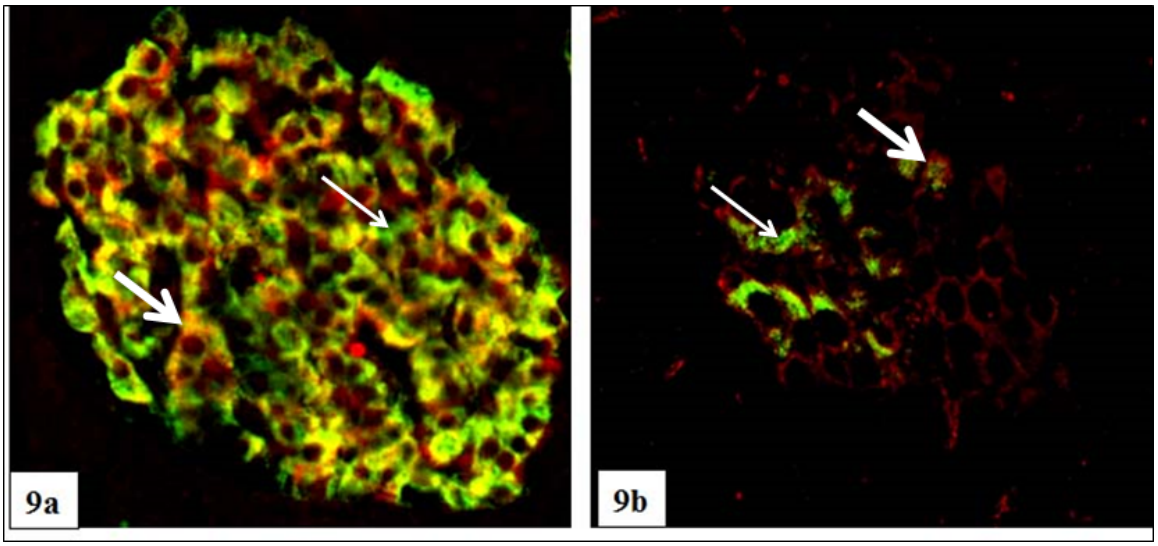

Figure 9: Immunofluorescence micrographs of nociceptin (red-thick arrows) and insulin (green-thin arrows) in the islets of non-diabetic (9a) and diabetic (9b) rats. Yellow colour indicates cells that contain both nociceptin and insulin. There is a large reduction in the number of nociceptin- and insulin-positive cells in diabetic rat. Magnification: X200

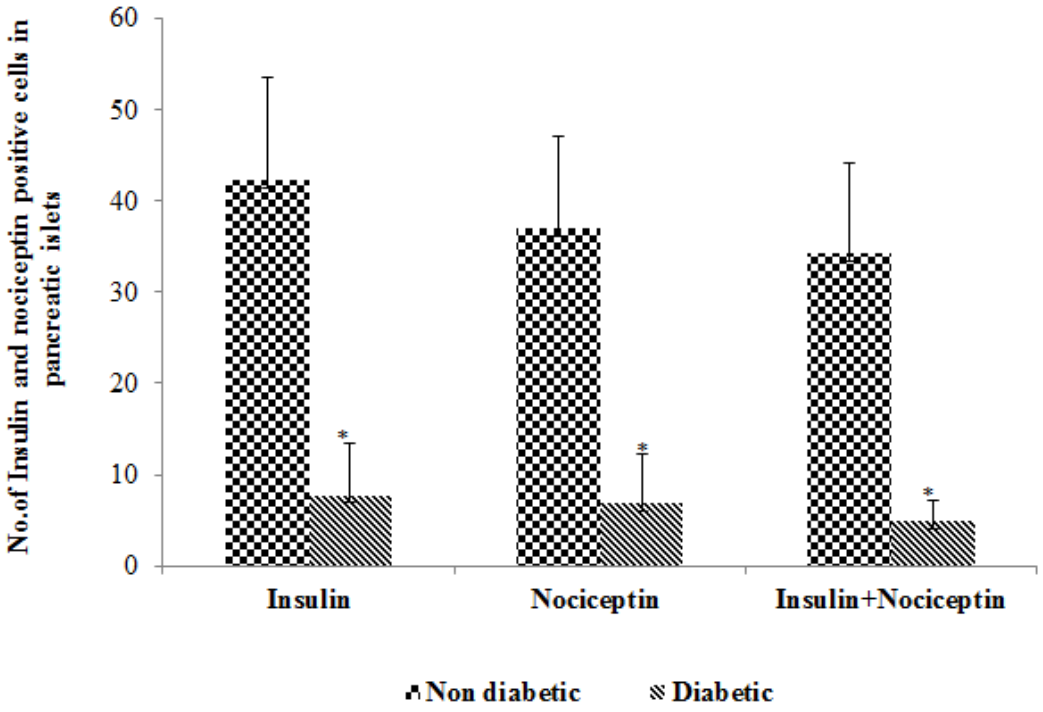

Figure 9c: Number of cells containing either insulin, nociceptin or insulin+nociceptin in pancreas of non-diabetic and diabetic rats.*p< 0.0000003 (Significantly lower compared to non diabetic rat pancreas).

DOI:10.14753/SE.2016.1887

4.1.7 Conventional electron microscopy

4.1.7.1 Non-diabetic and diabetic pancreatic β-cells

In non-diabetic pancreatic islets the most numerous cells were β -cells located in the central portion of pancreas that secrete insulin. Most part of the islet was covered with these cells. Insulin secretory granules of β-cells were observed with homologous electron dense inner core and surrounded by outer peripheral halo with crystalline matrix, which is electron lucent in nature. The secretory granules were almost polyhedral in shape with a pale matrix and have a rounded or slightly oval nucleus with irregular contour and narrow perinuclear cisterns. Non-diabetic β -cells contain numerous secretory granules which showed a mean diameter of 300 nm (Figure 10 A).

In diabetic pancreatic β-cell, the ultra-structural study revealed diverse degrees of injury in the β-cell organelles, such as alterations in the secretory granules with dialated halo space. Moreover the changes, like damage of chromatin matrix of the nucleus with dilated nuclear envelope, abundant vacoularization in cytoplasm were seen. Broken endoplasmic reticulum and swollen mitochondria with loss of cristae also were observed. Degenerated and sparsely scatted Golgi bodies contribute to explain the appearance of a diabetic syndrome in the rat tissue (Figure 10 B).

DOI:10.14753/SE.2016.1887

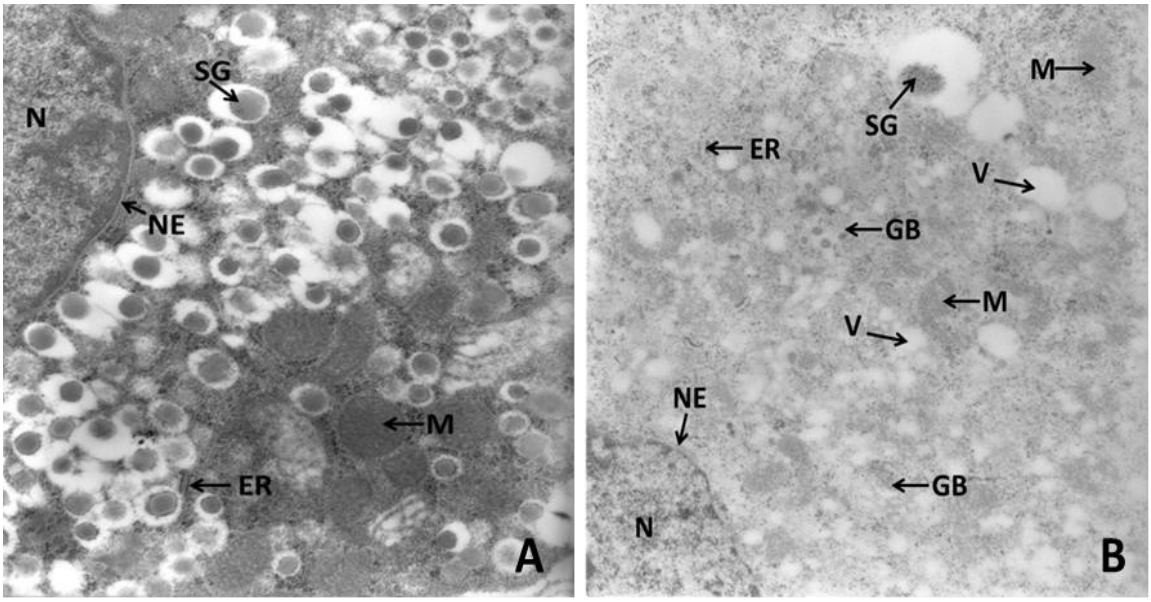

Figure 10: Non-diabetic rat (A) pancreatic β-cells showing a large number of secretory granules (SG), normal nuclear architecture (N), normal nuclear envelope (NE), normal endoplasmic reticulum (ER) and well developed mitochondria (M). In contrast, β-cells of diabetic rat (B) have fewer SG, ER, broken Golgi bodies (GB) and many vacuoles (V). (Magnification: X 14000)

DOI:10.14753/SE.2016.1887

4.1.7.2 Double labelling immunoelectron microscopic study

Immunoelectron microscopy was performed using two different sizes of immunogold particles to detect insulin and nociceptin in pancreatic islet cells. Insulin secretory granules of β-cells had a wide halo and a dense core of varying shapes (Figure 11a). Ultrastructural examination of the immunogold stained pancreatic islet cells clearly recognized two distinct types of gold particles on the granules of pancreatic β-cells. Immunogold particles conjugated with insulin (5 nm size) and nociceptin (10 nm gold particles) were observed on the secretory granules of β-cells. Glucagon secretory granules of α-cells remained unlabeled.

In diabetic rat pancreas, immunogold labeling of β-cells showed fewer secretory granules as compared to non-diabetic control (Figure 11b). Immunogold particles were not observed on other cell organelles, intracellular vesicles, connective tissue and other extracellular spaces.

Quantification of immunogold particles of nociceptin and insulin is shown in Figure 12. The mean ± SD of insulin-, and nociceptin-conjugated particles in non-diabetic islets was 78.33 ± 14.25 and 51.56 ± 12.98, respectively in comparison to diabetic islets (37.33 ± 5.70 for insulin; 20.44 ± 2.83 for nociceptin). The mean values were statistically significantly ($p < 0.000005$) different when analyzed by Mann-Whitney-U test. The number of insulin-, together with nociception-conjugated immunogold particles per field was lower after the onset of diabetes mellitus (129. 89 ± 23.33 vs. 57.78).

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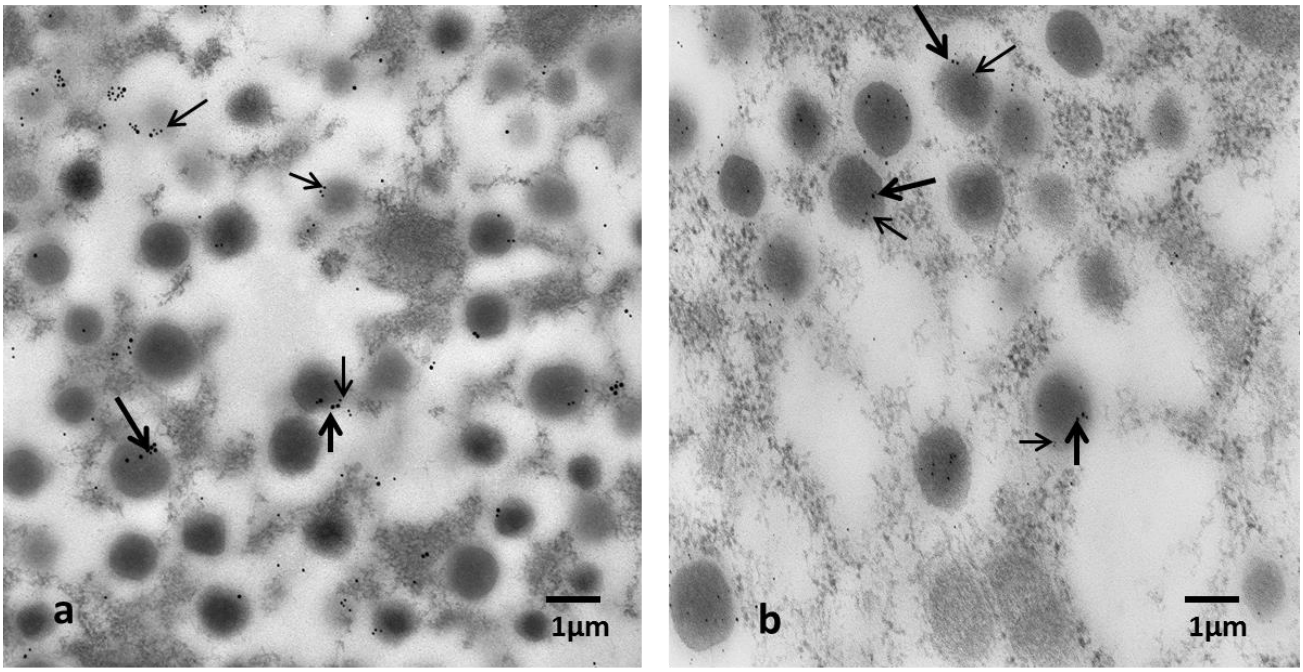

Figure 11: Immunoelectron microscopy of pancreatic β-cells of non-diabetic (a) and diabetic (b) rats. Secretory granules of pancreatic β cell contain immunogold particles labelled with insulin (5 nm, thin arrow) as well as nociceptin (10 nm, thick arrow). Note that the β-cells of diabetic rats contain fewer secretory granules. Scale bar = 1µm

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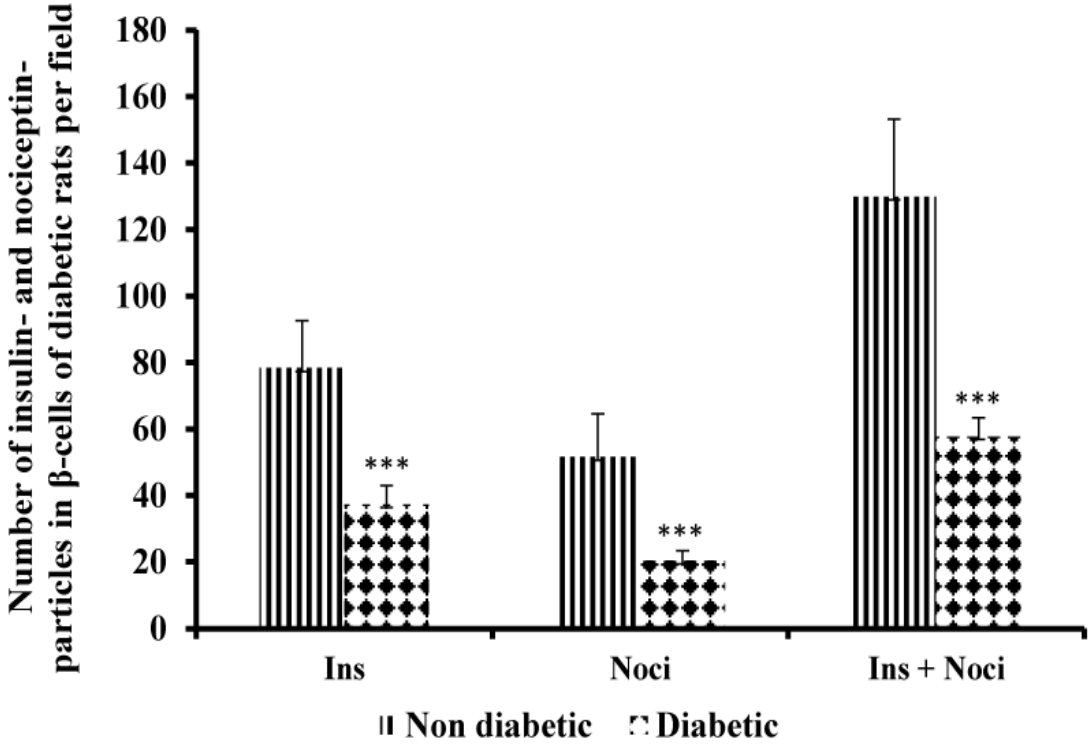

Figure 12 shows the total number of insulin (ins)- and nociceptin (noci)-conjugated particles in secretory granules of β-cells of non-diabetic control and diabetic rats. The number of insulin- and nociceptin-conjugated particles per field is significantly **(*****p < 0.00005) lower in β-cells of diabetic rats compared to non-diabetic control.

DOI:10.14753/SE.2016.1887

4.1.8 Western blot analysis

Semi-quantitative analysis of nociceptin in pancreatic tissues of non-diabetic and diabetic Wistar rats was performed to quantify the tissue content of nociceptin. It is evident from figure 13A that the expression of nociceptin was higher in the pancreas of non-diabetic compared with those of diabetic rats. Densitometric analysis is shown in figure 13B.

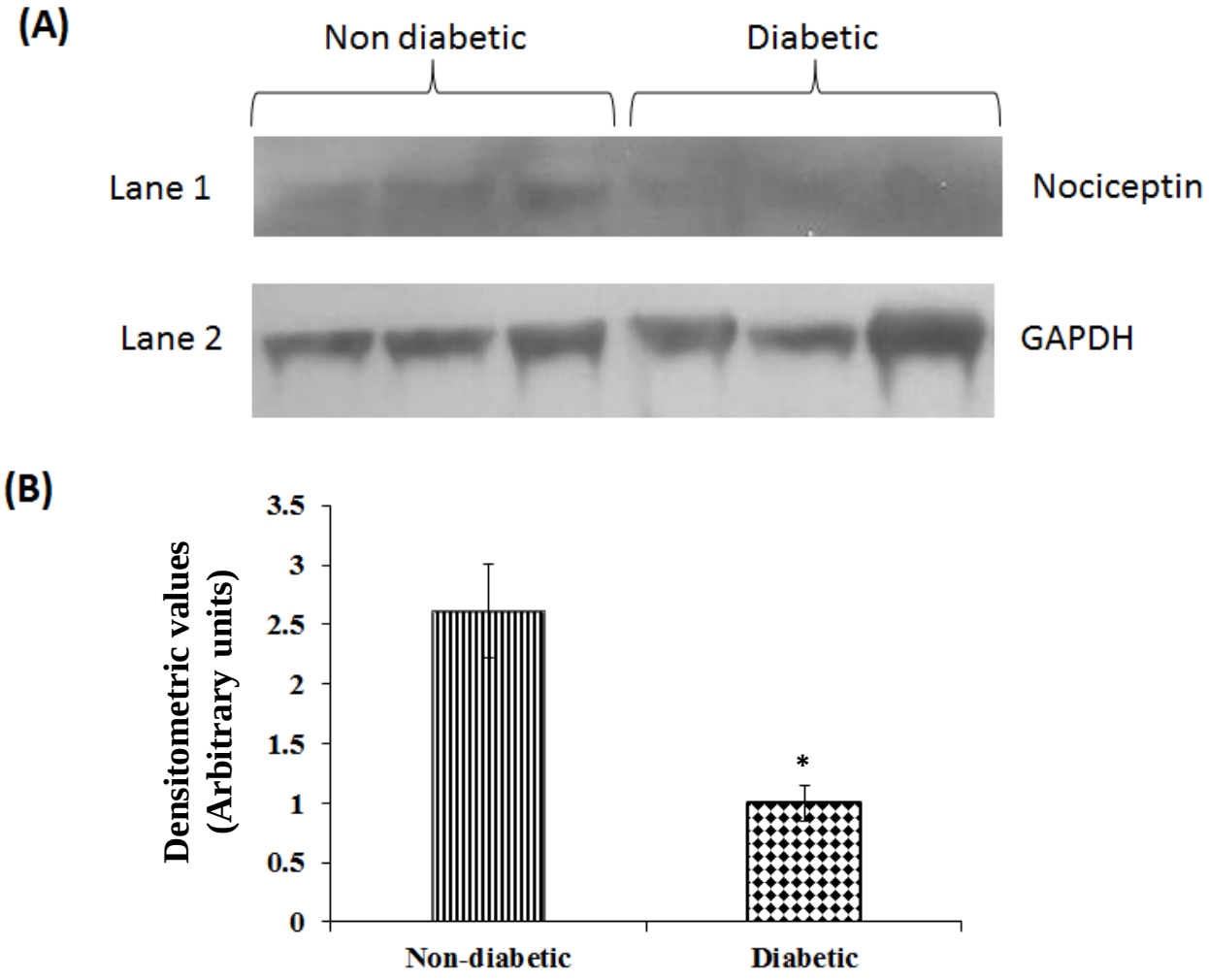

Figure 13A & B: Western blot analysis of nociceptin in the pancreas of non-diabetic and diabetic rats. Lane 1 shows a reduced nociceptin concentration in the pancreas of diabetic rats compared to non-diabetic control and GAPDH loading control (Lane 2). **13B:** Densitometric analysis (arbitrary numbers) of the level of nociceptin in the pancreas of non-diabetic compared to diabetic rats. Note that nociceptin is significantly ($* p < 0.05$) lower in the pancreas of diabetic rats compared to non-diabetic controls.

4.2. Uterus

4.2.1 Body and organ weight ratios

The average body weight of the non-diabetic female rats was found to be 201 ± 18 g. Uterus to body weight ratio for non-diabetic rats was 0.0027 ± 0.0005. The average body weight of diabetic rats was 168 ± 23g and uterus to body weight ratio was 0.0015±0.0003. Statistical analysis using Mann-Whitney U test reveals no significance.

4.2.2 Glucose measurement

The random blood glucose level was measured for each individual rat of both groups. The average blood glucose level for non-diabetic controls was 109 ± 18 mg/dL. The average blood glucose level for diabetic rats was 576 ± 26 mg/dL. There was a marked ($p < 0.05$) difference between the glucose level in non-diabetic controls versus diabetic rats.

4.2.3 Glucose tolerance test of female rats

Figure 14 shows the changes in glucose levels during intraperitoneal glucose tolerance test (IPGTT). The glucose load of 2g/kg body weight, when given intraperitoneally resulted in different responses by non-diabetic control and STZ-diabetic groups. Basal plasma glucose was significantly higher in the diabetic female rats after the i.p. glucose load, however, the handling of glucose was significantly ($p <0.001$) poorer in diabetic rats compared to non-diabetic controls.

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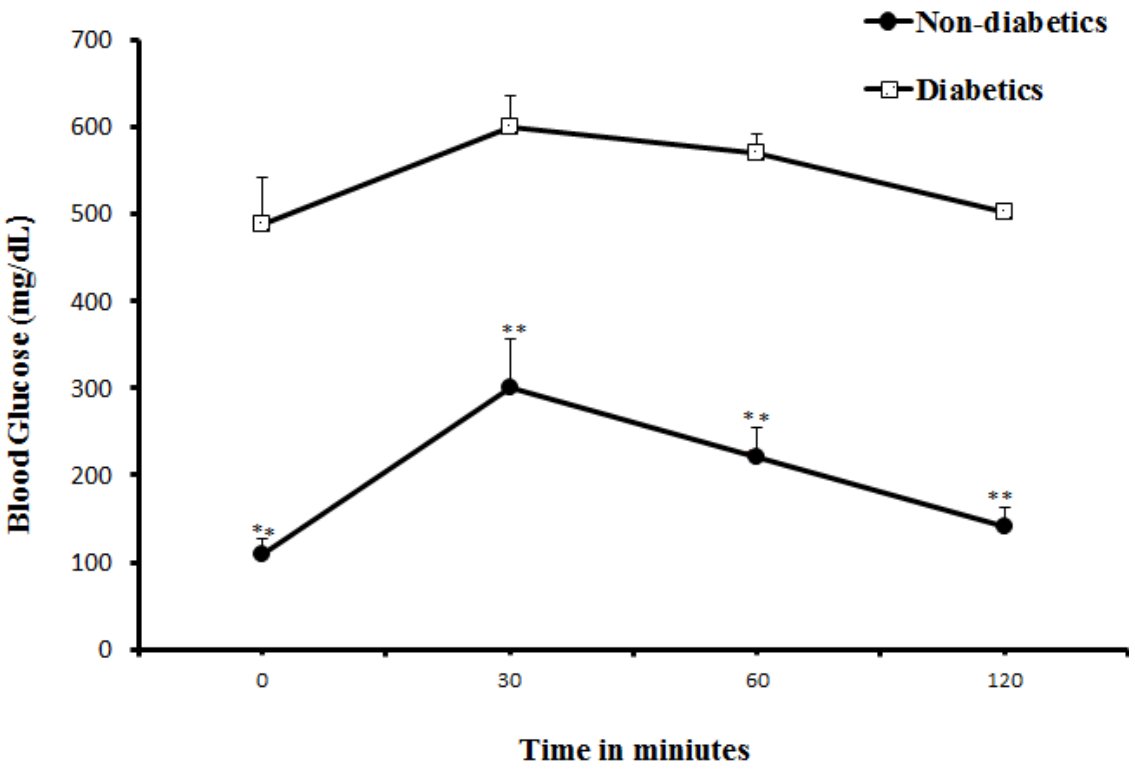

Figure 14: Glucose tolerance test of non-diabetic and STZ-induced diabetic female rats

** Significantly ($p < 0.001$) lower compared to diabetics.

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4.2.4 Gross morphology of the uterus

The uteri of diabetic rats were smaller compared to non-diabetic controls as early as 15 days after the onset of DM. In addition, the number of blood vessels supplying the uteri of diabetic rats appeared to be fewer in number when compared to non-diabetic control (Figure 15).

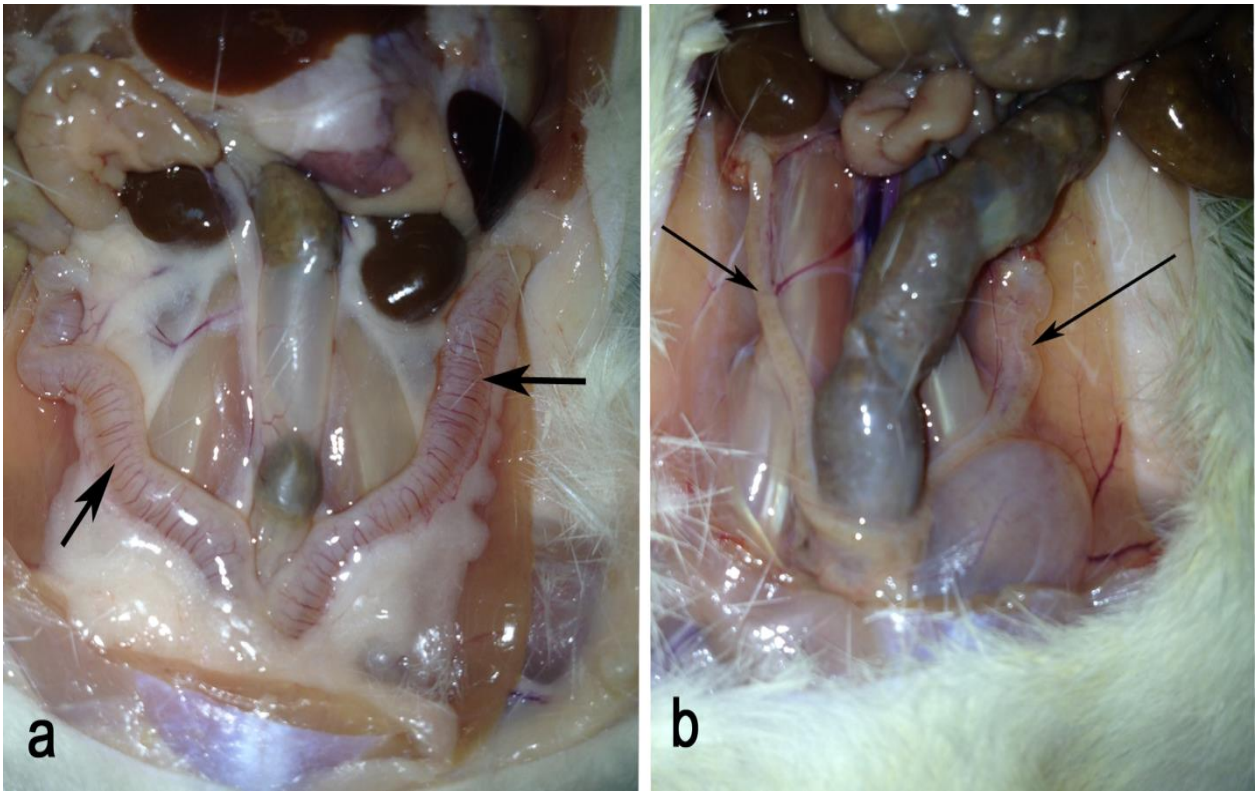

Figure 15: Gross morphology of non-diabetic control (a) and diabetic (b) rat uteri. Thick arrows show the non-diabetic and thin arrows diabetic. Note that the density of blood vessels in non-diabetic rat uterus appears to be more conspicuous compared to that of diabetic. Magnification: X2

DOI:10.14753/SE.2016.1887

4.2.5 Light microscopy study

Light microscopy study was also carried out on semithin sections of uterus to examine the morphology of the rat uterus after the onset of DM. The endometrium and myometrium of the uterus of non-diabetic control were significantly thicker than those of diabetic rats (Figure 16). Moreover, the endometrial glands were less prominent in diabetic rats when compared non-diabetic control rats. The overall size of the uterus was much smaller in diabetic rats compared to control (Figure 17)

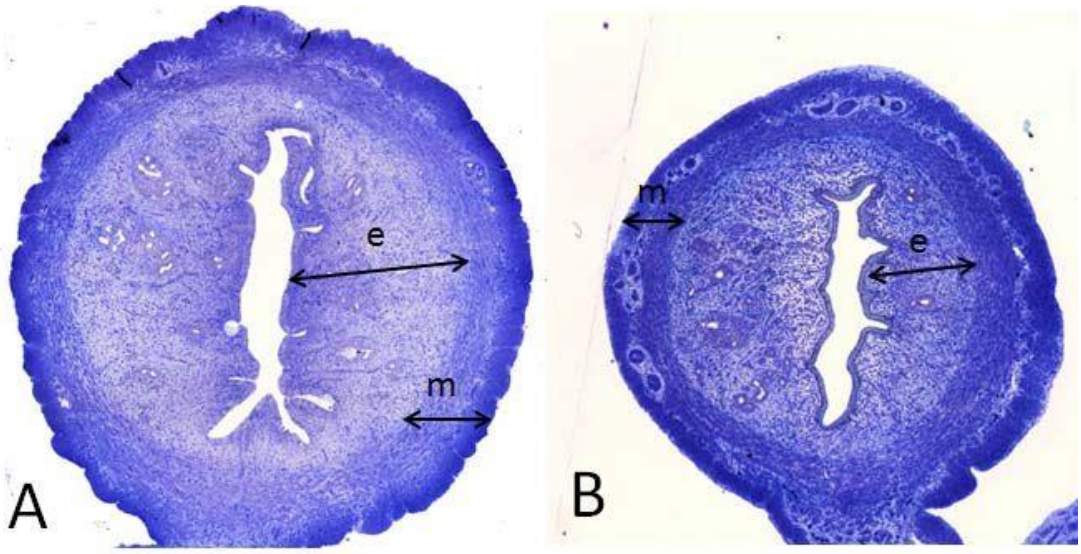

Figure 16: Representative micrographs of non-diabetic (A), and diabetic (B) rat uterus 2 weeks after STZ treatment. n=6, m= myometrium, e= endometrium. Magnification: X 40.

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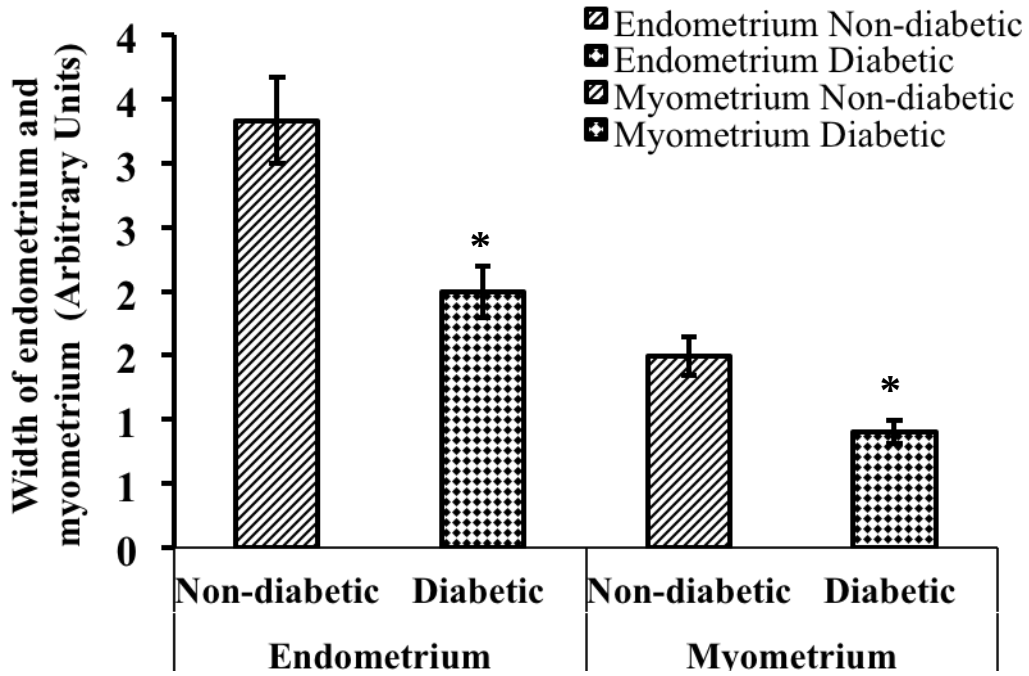

Figure 17: Width of endometrium and myometrium layers of uterus in non-diabetic and STZ-diabetic rats.* shows statistical significance at $p < 0.05$.

4.2.6 Immunofluorescence study

Six uterine tissue sections from the non-diabetic control and diabetic rats were examined with confocal microscope and images were taken with EZ-C1 software (Eclipse 80i, Nikon Japan). Figure 18 show that nociceptin is localized in the uterus of both non-diabetic and diabetic rats. Nociceptin coupled with FITC (green) immunofluorescence staining was rarely seen in the perimetrial layer of the uterus. However, large number of nociceptin-positive cells was observed in the endometrial layer of the uterus.

The number of nociceptin-immunoreactive cells was significantly ($p < 0.05$) lower in the endometrium of diabetic rats compared to that of non-diabetic control (Figure 19). In addition, the columnar epithelium of the endometrium of diabetic rat uterus did not express nociceptin as strongly as that of non-diabetic control.

The number of nociceptin-immunoreactive cells seen in the myometrium of diabetic rats was significantly ($p < 0.05$) lower compared to that of non-diabetic control group. (Figure 19). Moreover the myometrium of the uterus of both non-diabetic control and diabetic rats contain nociceptin-immunoreactive neuronal cell bodies and nerve fibres. These nerve fibres are varicose in nature. The density of the nociceptinergic innervation of the myometrium of diabetic rats was less pronounced when compared to that of non-diabetic control.

4.2.7 Morphometry

Morphometric evaluation of immunofluorescent images shows that the number of nociceptin-positive cells was significantly ($p < 0.05$) reduced after the onset of diabetes (Figure 19)

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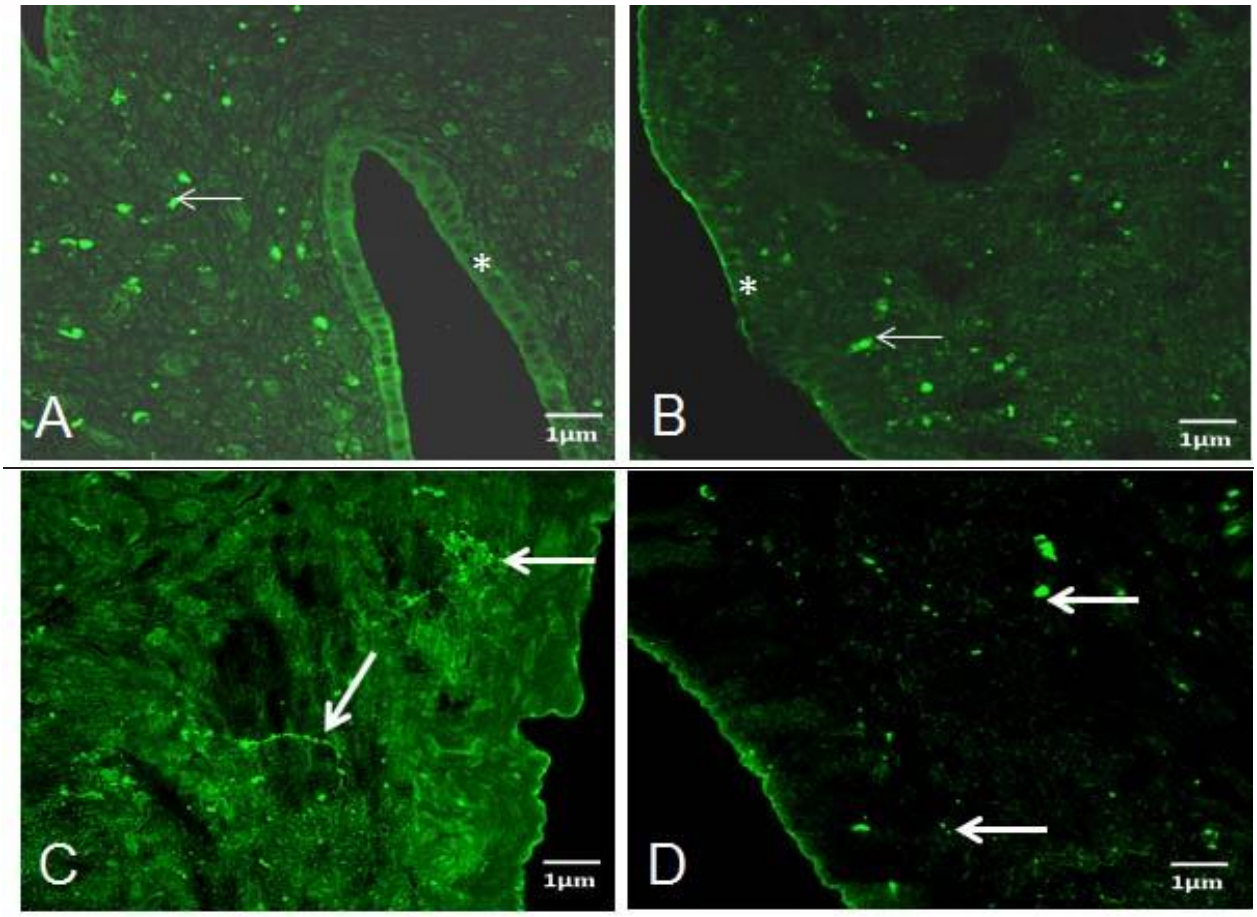

Figure 18: Immunofluorescence micrographs of nociceptin-positive cells (thin arrow) in the endometrium of non-diabetic (A) and diabetic (B) rats after the induction of diabetes. Note that the number of nociceptin-positive cells (thin arrows) is more in the endometrium of non-diabetic compared to that of diabetic rats. Moreover, the expression of nociceptin is more intense in the compact layer (*) of the endometrium of non-diabetic compared to that of diabetic rats. Nociceptin-immunoreactive varicose nerves (thick arrows) are more abundant in the myometrium of non-diabetic (C) when compared to that of diabetic (D) rats. Scale bar = 1 µm.

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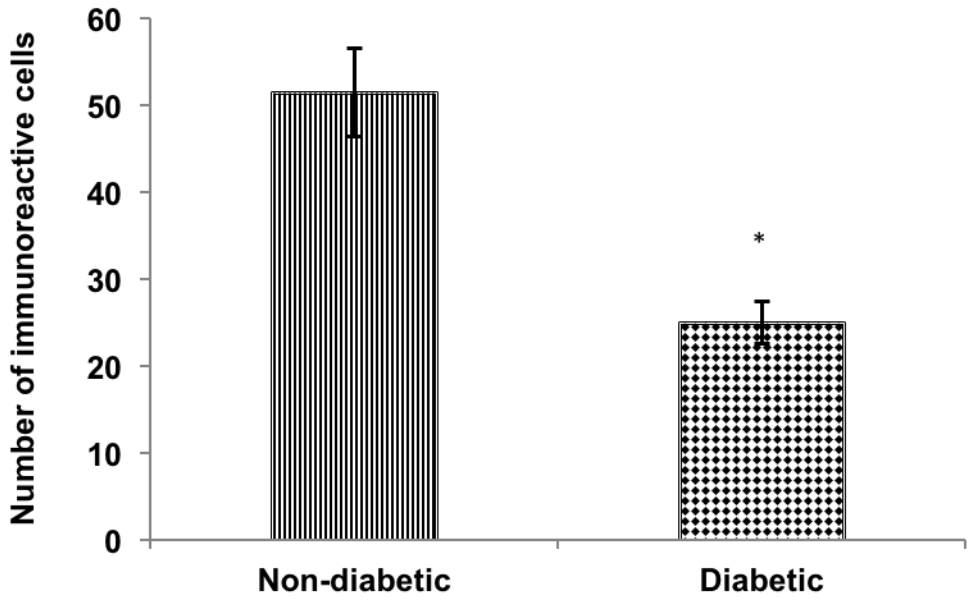

Figure 19: Morphometry of nociceptin immuno-positive cells in the endometrium and myometrium of the uterus of non diabetic control and diabetic rats. Note that the number of immuno-positive cells were significantly ($p < 0.05$) reduced after the onset of diabetes. n= 6

DOI:10.14753/SE.2016.1887

4.2.8 Western blot analysis

Western blot analysis of nociceptin in the uterus of non-diabetic control and diabetic rats shows a reduced nociceptin concentration in the uterus of diabetic rats compared to control. Densitometric analysis shows that the level of nociceptin in the uterus of diabetic rats is about half of that of non-diabetic control rats (Figurre 20A and B).

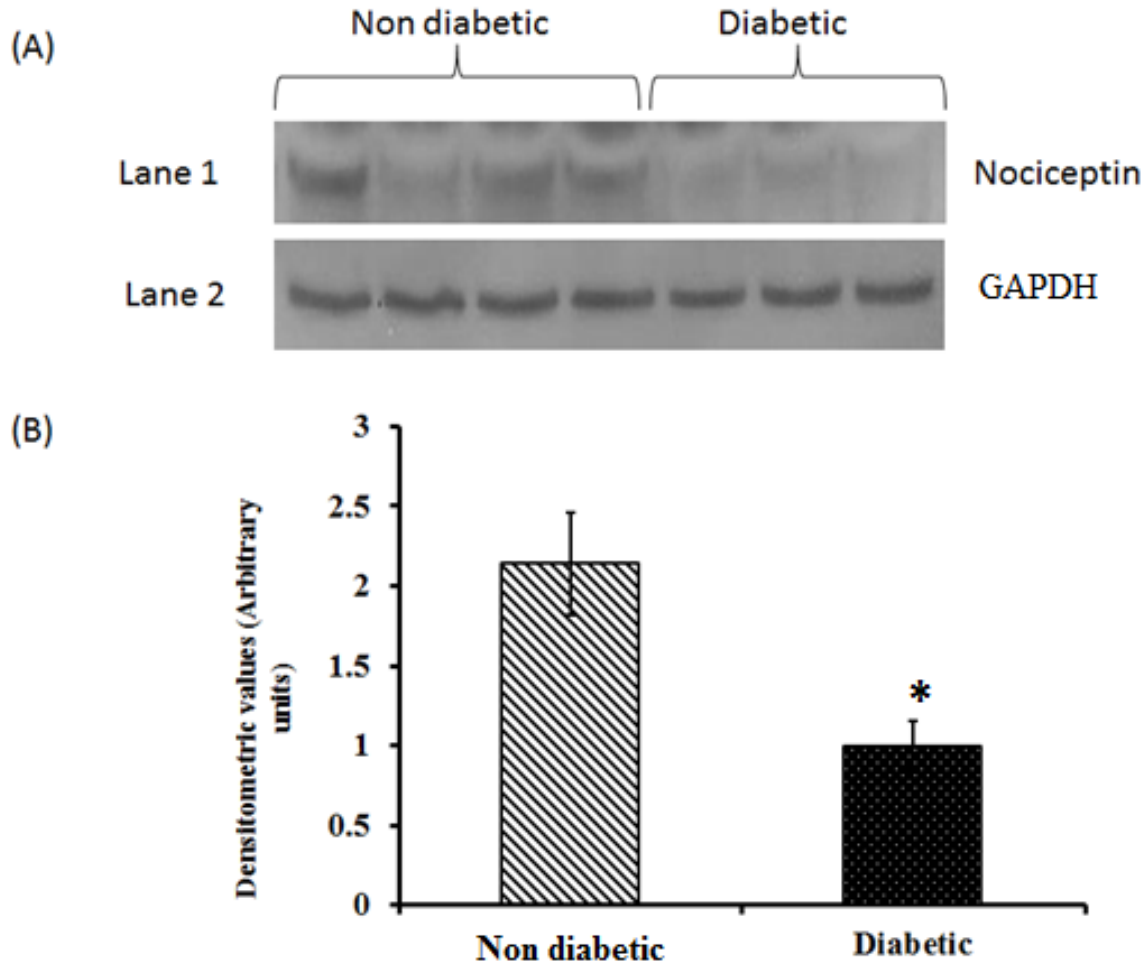

Figure 20: Western blot analysis of nociceptin in the uterus of non-diabetic control and diabetic rats **(A)**. Lane 1 shows a reduced nociceptin concentration in the uterus of diabetic rats compared to non-diabetic control and GAPDH loading control (Lane 2). Densitometric analysis (arbitrary numbers) of the level of nociceptin in the uterus of non-diabetic control compared to diabetic rats **(B)**. Note that nociceptin is significantly ($* p < 0.05$) lower in the uterus of diabetic rats compared to non-diabetic controls.

DOI:10.14753/SE.2016.1887

4.2.9 Conventional electron microscopy of uterus

Endometrium

Uterine tissue samples from the non-diabetic control and diabetic rats were processed for electron microscopy to determine whether the ultrastructure of the uterus is altered after the onset of diabetes. Electron microscopy study revealed abnormal nuclei in columnar epithelial layer of the endometrium of diabetic rats compared to that of non-diabetic control rats.

Moreover, large, dense-cored vesicles and dark granules localized to the columnar epithelial cells of the endometrium of the uterus of diabetic rats are more numerous compared to that of non- diabetic control. Epithelial cells of the endometrium of the uterus of normal rats contain significantly more cilia, in contrast to the epithelial cells of the diabetic rat uterus (Figure 21).

Myometrium

Many more degenerated smooth muscle cells with pyknotic nuclei were observed in the myometrium of the uterus of diabetic rats when compared to non-diabetic control. The myocytes of the uterus of diabetic rats were associated with pleomorphic and shrunken nuclei with large areas of heterochromatin compared to non-diabetic control. The myocytes of diabetic rat uteri contain fewer and disorganized myofibrils as compared to the non-diabetic control. The myometrium of diabetic rats also contained large number of “spaces” due to degenerated myocytes (Figure 22).

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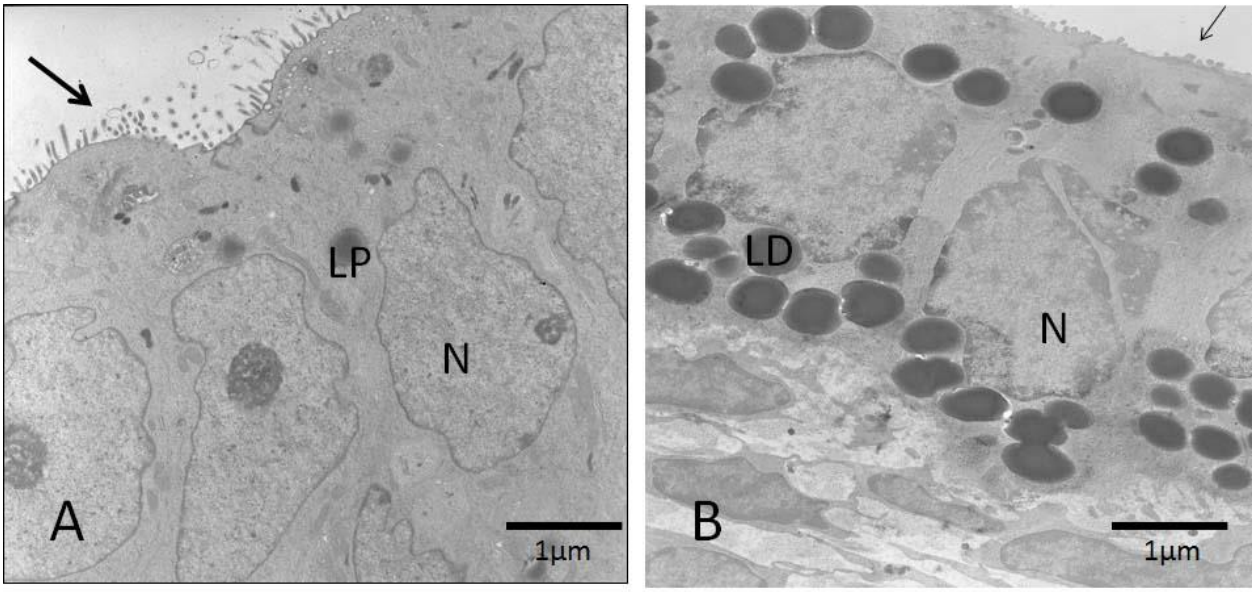

Figure 21: Micrographs of the endometrium of rat uterus of non-diabetic control (A) and diabetic (B) rats. Note the large, dense lipid droplets (LP) in the epithelial cells of the endometrium of the uterus of diabetic rats. The number of cilia (thin arrows) on the epithelial cells of the uterus of diabetic rats decreased significantly after the onset of diabetes. Scale bar= 1µm

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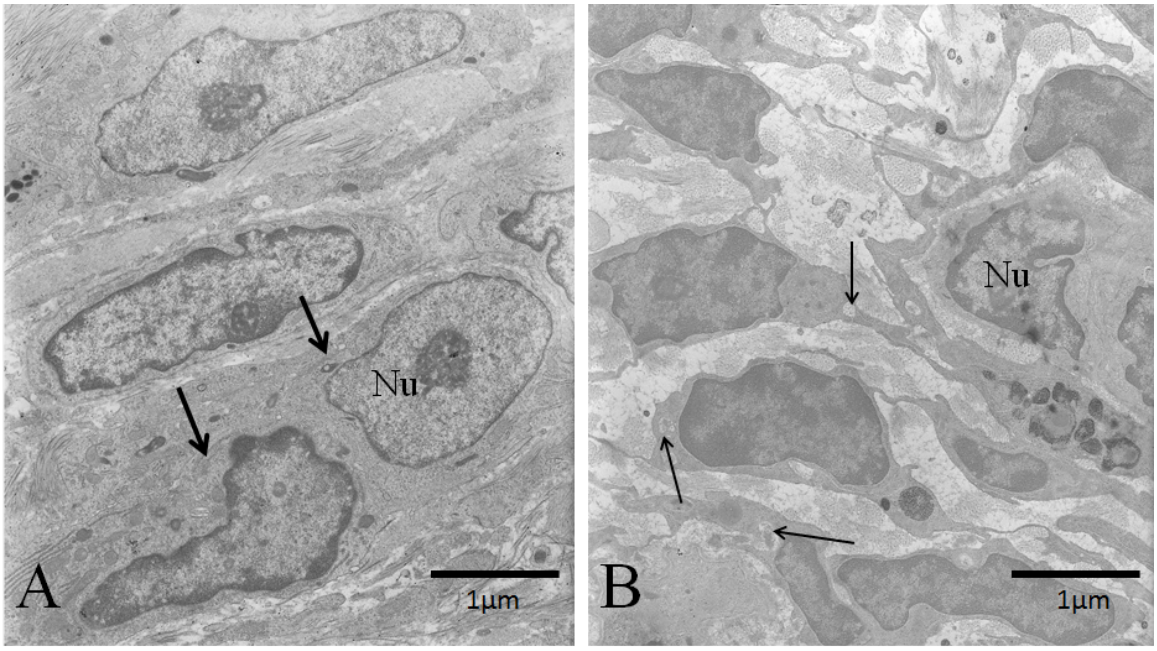

Figure 22: Micrographs of the myometrium of rat uterus of non-diabetic control (A) and diabetic (B) rats. The nuclei (Nu) of the myocytes in the uterus of diabetic rats (B) were pleomorphic and contained large areas of heterochromatin dispersed throughout the nuclei compared to non-diabetic control (A). The myocytes of diabetic rats uterus contain fewer myofibrils (thin arrow) when compared to the non-diabetic control group (A). Scale bar =1 µm

DOI:10.14753/SE.2016.1887

4.2.10 Immunoelectron microscopy study

Ten nanometer nociceptin-immunopositive gold particles were observed in the myometrium of both non-diabetic control and diabetic rats. nociceptin was discerned mainly on the myofibrils in longitudinal smooth muscle sections. In circular muscle cells, the colloidal gold particles localization for nociceptin was also noticed. The gold particles were not seen on other cell organelles such as the mitochondria, Golgi apparatus, endoplasmic reticulum and plasma membrane (Figure 23).

In diabetic rat myometrium, nociceptin-labelled colloidal gold particles were also observed on the myofibrils of smooth muscle cells. The number of nociceptin-immunoreactive gold particles was much lower in diabetic rat myometrium compared to non-diabetic control.

Morphometric evaluation of the sections showed a significant ($p < 0.05$) reduction in the number of nociceptin-labelled immunoparticles in the myometrium of diabetic rats compared to non-diabetic control (Figure 24).

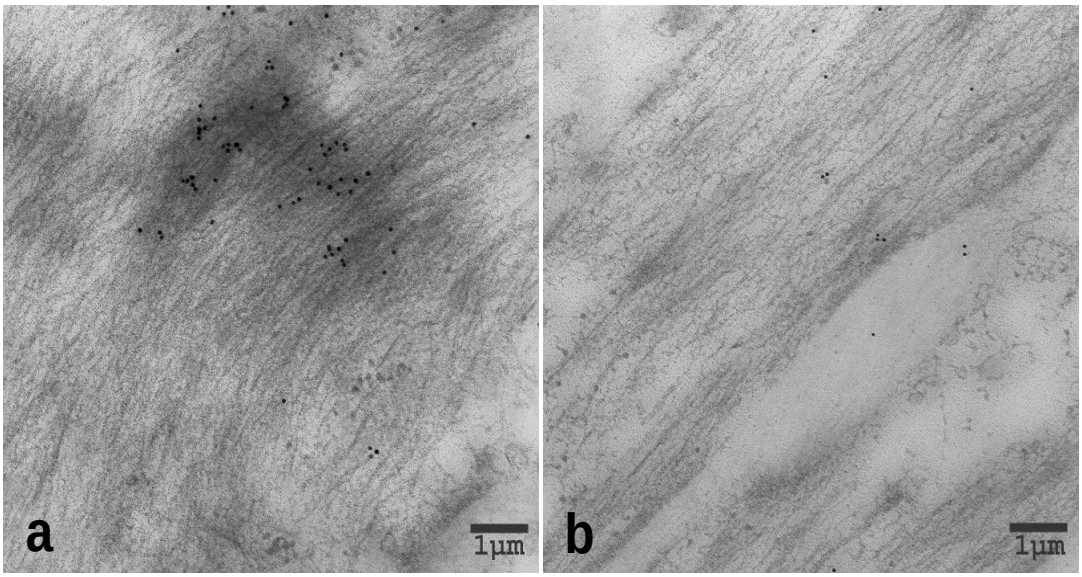

Figure 23: Micrographs of nociceptin-immunoreactive gold particles in the myocytes of the myometrium of non-diabetic (a) and diabetic (b) rats uterus.

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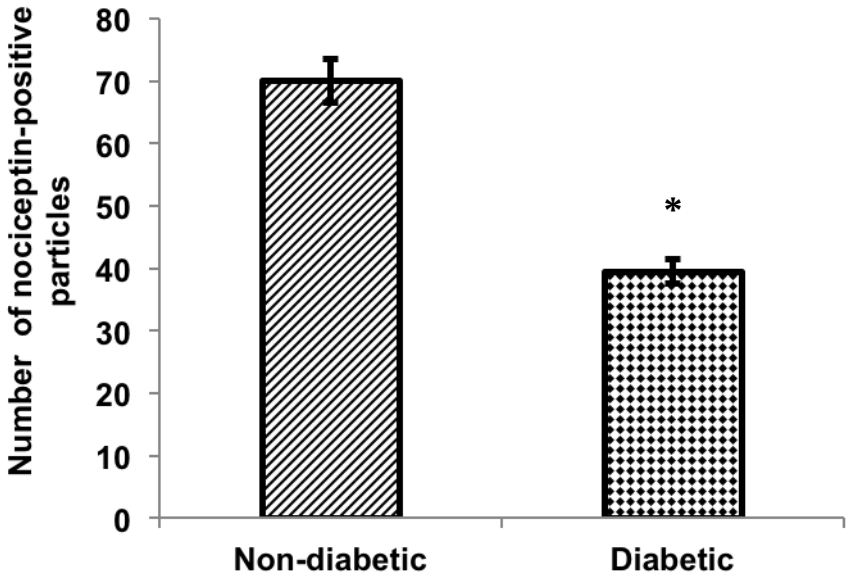

Figure 24: Number of nociceptin-positive immunogold particles in myometrium of non-diabetic control and diabetic rat uterus. Note that the number of nociceptin-positive immunogold particles is significantly lower ($p < 0.05$) in diabetic rat myometrium compared to non-diabetic control. n=6.

DOI:10.14753/SE.2016.1887

5. Discussion

This study investigated the presence of nociceptin in two organ systems, namely, the pancreatic islet of Langerhans (an endocrine organ) and the uterus (a female reproductive organ) by light microscopy, immunohistochemistry, Western blot and electron microscopy, that have never been studied earlier. The study design included two groups of Wistar rats, non-diabetic control and STZ- induced diabetic rats. Blood glucose was measured one week after injection of STZ. Two weeks after injection of STZ, pancreatic and uterine tissue samples were collected for light microscopy, immunohistochemistry, Western blot and electron microscopy. Animal body weight, pancreas and uterine weights were also taken. Glucose tolerance test was conducted after i.p injection of glucose. Morphological observation of non-diabetic control and diabetic uterus was carried out.

The STZ animal model of diabetes was used in this study. It is believed that STZ-induced diabetes mellitus in adult animals is similar to that of type 1, previously known as insulin dependent diabetes mellitus. The streptozotocin (STZ) induced diabetes mellitus is widely used and generally accepted as an animal model of diabetes. In our experiments it was used to measure the different symptomatic parameters related to diabetes in the body, organs and tissues.

Ganda et al.[141] reported that STZ induces necrosis in pancreatic β-cells and damages mitochondria. According to Matkovics et al.[142] STZ is a nitric oxide donor (NO) induces DNA strand breakdown and also DNA methylation through the formation of cabonium ions ($CH3^{+}$) which results the destruction of pancreatic β-cells.

STZ also induces cell necrosis in the pancreatic islet by intracellular nicotinamide adenine dinucleotide (NAD) depletion [143]. Kröncke et al. [144] showed that nitric oxide is formed soon after STZ is degraded intracellularly.

The induction of type 1 diabetes mellitus by this chemical, in which the disruption of pancreatic β-cells occurs causes a sharp decrease in the synthesis of insulin [94]. Some studies revealed that the degree of reduction in body weight is significantly higher in male rats compared to female rats [145].

5.1 Metabolic parameters

Body weight

The animal body weight of non-diabetic rats increased steadily over time in comparison to STZ-induced-diabetic rats, which decreased with time. Our result is in line with those reported in the literature. It is well known that diabetes mellitus is associated with a loss of body and organ weight [94]. The reason for the loss of body weight is not clear but it may be due the consumption of proteins instead of glucose, since glucose uptake is impaired in diabetes mellitus. It is therefore not available to cells for consumption. STZ as an alkylating agent is irreversibly damaging kidney function as well and consequently results in extreme urination.

Blood glucose level and Glucose tolerance test

Blood glucose level was significantly higher in diabetic rats compared non-diabetic controls. This observation corroborates other reports in literature [146]. Glucose tolerance test in non-diabetic rats revealed a rapid onset of glucose level followed by decline over time. In diabetic rats, which already have high level of blood glucose, the glucose challenge caused an even higher level of blood glucose after intraperitoneal injection of glucose. In contrast to non-diabetic controls, the blood glucose level of diabetic rats did not return to normal value even after 120 min.

5.2 Pancreas

The pancreas consists of an exocrine and an endocrine part. The endocrine pancreas also known as islets of Langerhans secretes glucagon, insulin, somatostatin and pancreatic polypeptide and probably neuropeptide, like neuropeptide-Y [147]. Some of the neuropeptides coexist with islets. For example, insulin and islet amyloid polypeptide or amylin (IAPP) are known to be co-stored in the same secretory granules and co-released at secretion. However, the two encoding genes may not always be co-regulated. Similarly, Adeghate [128] reported the distribution of several neuropeptides and islet peptides in the pancreas of normal and diabetic rats. Neuropeptides from the endocrine pancreas (the islets of Langerhans) play an important role in the regulation of blood glucose levels. So far,

there are few neuropeptides isolated and sequenced from the endocrine pancreas. *In situ* hybridization and immunocytochemical techniques have been used to demonstrate the occurrence of peptides in the pancreas[148]. Nerve fibers present in the pancreatic tissue contain a wide array of biologically active substances, particularly neuropeptides, which mainly serve as important regulators both to the exocrine and endocrine part of the organ. Although considerable efforts have been undertaken, the role of regulatory peptides on pancreatic activity is not fully understood [149]. In addition, β-cells express many neuropeptides during development and under pathophysiological conditions [150].

With this in mind, we developed a hypothesis that, nociceptin, as a neuropeptide is present in the endocrine pancreas. Our study shows that nociceptin is present in the islet of Langerhans. Since it has been shown that pancreatic islet harbours many neuropeptides, it is not surprising, but a new result, that nociceptin was localized in the pancreatic islet cells. The pattern of distribution of nociceptin in islet cells was found similar to that of insulin. In addition, the pattern of distribution of nociceptin was shown completely deranged after the onset of DM. The reason why the distribution of nociceptin is altered in DM is not clear. However, it has been shown that, the expression of other neuropeptides may be altered in pathological conditions. For example, neuropeptide Y, that was undetectable in the cells of normal adult rats, was re-expressed in insulinomas cells [151]. Similarly, in STZ-induced type-1 diabetic rats, the number of neuropeptide-Y expressing cells was higher in the islet cells compared to non-diabetic control [151].

Western blot study was undertaking to further ascertain and confirm our immunohistochemical and immunofluorescence findings of the presence of nociceptin in the pancreas. Western blot study confirmed that nociceptin is indeed present in the pancreas, and the pancreatic tissue level is reduced after the onset of diabetes. We could not compare our results with that of the literature since this is the first study to quantify the tissue level of nociceptin in the pancreas.

The localization of nociceptin to the endocrine cells of the pancreas and its pattern of distribution that resemble that of insulin prompted us to examine a potential degree of co-localization of nociceptin with pancreatic hormones. Double-labelling immunofluorescence

was used to determine whether nociceptin co-localizes with any of the pancreatic hormones and if so, what happens to the co-localization after the onset of DM.

Immunofluorescence study showed that nociceptin co-localizes with insulin in both non-diabetic and diabetic rat pancreas. However, the degree of co-localization is significantly reduced in DM. Since this is the first description of nociceptin in the endocrine pancreas it is difficult to compare our results with that of the literature. Immunoelectron microscopy study confirmed our immunofluorescence result of the presence of nociceptin with insulin in pancreatic β-cells. It was interesting to observe that nociceptin was localized to secretory granules of the pancreatic β-cells and was not seen in other cytoplasmic organelles. The reason for this needs further analysis. It is possible that the main function of nociceptin in the pancreas is to regulate insulin metabolism. Further studies will be required to elucidate the precise role of nociceptin in pancreatic β-cells.

The present study reveals the co-existence of nociceptin and insulin in β-cells of the pancreas. Immunogold double staining technique with transmission electron microscope application was effective in identifying gold particles conjugated to nociceptin and insulin. Immunogold detection of co-localized peptides has been used in several studies,[152, 153] which allows the immunolabelling of two different antigens. Tapia et al. [154] suggested that double immunogold staining technique may provide useful information on neuroendocrine cell dynamics in normal and diseased states.

A search of the literature shows that this is the first study that shows the presence of and co-localization of nociceptin with insulin in the endocrine pancreas. The possibility of coexistence of peptides derived from different precursors has been reviewed by Larsson [155]. A large number of bioregulatory agents has been reported to co-localize with hormones and neurotransmitters in endocrine and neural cells, respectively [133, 148]. Wierup et al. [150] reported that cocaine- and amphetamine-regulated transcript, co-expresses with somatostatin in pancreatic islets. Nociceptin is reported to be expressed mainly in the central as well as peripheral nervous systems [77]. It has also been demonstrated in non-neural organs such as the major salivary glands of some mammals including rats and cow [156].

Why is nociceptin localized to pancreatic islet cells? The other question also arrises: why is nociceptin co-localized with insulin? To answer this question needs further functional studies. However, nociceptin may play a role in the metabolism of insulin either in an autocrine or paracrine manner. It may also inhibit or even stimulate insulin release from pancreatic β-cells. Therefore, further may be needed to elucidate the exact role of nociceptin in the endocrine pancreas.

Our study showed a large and marked reduction in the number of nociceptin-containing cells in the islet cells in diabetic rats compared to non-diabetic controls. It is not clear why the tissue level of nociceptin decreases in diabetes? It may be due to reorganization of cells within an organ. Moreover, De Paul et al. [157] showed that the cells of the pituitary gland, for example, undergo extensive alterations in different physiological and experimental conditions. The changes in the tissue level of nociceptin may have similar function.

The level of nociceptin in diabetic rats has been examined by Tekes et al. [79]. They used radioimmunoassay technique to measure the level of nociceptin in the plasma and CSF of normal and diabetic rats. They observed no difference in nociceptin level between non-diabetic control and diabetic rats, but Liu et al. [80] showed that nociceptin level is increased in the CNS of diabetic neuropathy pain model of rats. Our study using Western Blot technique, however, showed that nociceptin is reduced significantly in diabetic rat pancreas when compared to non-diabetic control. The reason for this discrepancy is not clear. The difference in the techniques and the samples used may have contributed to the discrepancy noted in the literature and the current study. For example, Tekes et al. [79] and Liu et al. [80] examined the concentration of nociceptin in the plasma and CSF but we determined the level of nociceptin in pancreatic tissue.

The exact role of nociceptin in the regulation of hormones of the pancreas is not clear. However, it is not impossible to attribute a role for nociceptin in the secretion of pancreatic hormones. For example, it has been shown that endogenous opioids like Leu-Enkephalin can induce large and significant increases in insulin and glucagon secretions from normal rat pancreas [158]. In fact, Matsushita et al.[85] showed that chronic cerebroventricular infusion of nociceptin increased plasma insulin level significantly. Other actions of

nociceptin on carbohydrate metabolism include its ability to reduce preference for high sucrose diet [159].

All of these observations support the view that nociceptin may have a physiological role in insulin secretion.

5.3 Uterus

Gross morphology

Our study showed that the uterus of diabetic rats was significantly reduced in size compared to non-diabetic control rats. A possible reason for the reduction in the size and weight of the uterus is not completely clear. However, previous studies have shown a significant reduction in the size of the uterus of diabetic rats [160]. The possible reasons for the reduction in the body weight of diabetic rats may be applicable to the reduction in organ weight observed in this study. In addition, we observed that the density of blood vessels supplying the uterus is reduced after the onset of STZ-induced diabetes. This may be due to diabetes-induced lesion in blood vessels. As concluded by Garris et al. [161], impaired blood vessels will dimish blood flow into the organ and the mass of the organ may also be affected.

Light microscopy

The reduction in uterine size prompted us to examine the different layers of the uterus to determine whether, alterations in the width of the organ will occur after the onset of diabetes. Light microscopy study revealed that the width of both the endometrium as well as the myometrium as early as 15 days after the onset of diabetes was significantly reduced. This morphological observation confirmed and showed that DM has detrimental effect on the rat uterus. The reason for this uterine sensitivity to DM is not clear. It may be due to the fact, that the uterus uses a large quantity of glucose. Uterine tissue may therefore be under severe control by alterations in the absence of insulin which promotes glucose uptake in cells.

The atrophy of the female reproductive tract has been reported in db/db mice [161]. In addition, DM was also shown to affect the assembly of cytoplasmic organelles in the smooth muscle cells [162].

Immunofluorescence

The existence of endogenous peptides and opioid receptors in non-neural tissues, namely placental [163, 164] and uterine tissue [165] were demonstrated where they have been implicated in the regulation of reproductive organs [163–166]. Binding sites for opioid receptor antagonist [167] have been identified in rat uterine membranes and are subject to down-regulation during gestation [168]. DAMEA ([D-Ala-2, D-met 5] enkephalin), an analogue of met-enkephalin increases the duration of spontaneous contractions of myometrium in rat [169].

As indicated earlier, nociceptin plasma level changes in several animal models or human diseases, including the female fibromyalgia syndrome [170] and postpartum depression [88]. A high expression of nociceptin in adenomyosis may contribute to the pathogenesis of dysmenorrhea [171]. The nociceptin–NOP receptor system controls sexual receptivity and manages reproductive behaviour and physiology through actions in the limbic system and hypothalamus [172]. A number of endogenous peptides participate in the regulation of uterine contractility, including oxytocin and tachykinin stimulating contractions [173–176]. Other neuropeptides, such as calcitonin gene-related peptide (CGRP), also contribute to relaxation of the uterine smooth muscle [177–179]. CGRP and tachykinin are confined to the capsaicin-sensitive sensory nerve endings.

In view of the fact that cells in the uterus contain several neuropeptides and neurotransmitters, it is not surprising that our study showed that nociceptin is also present in both the endometrium and myometrium of the uterus nociceptin was shown to be present in the endometrial stroma and in the myometrium as well. The role of nociceptin in uterine function has not been clearly elucidated, however, it has been implicated in labour, which is associated with extreme pain. Many studies including those of [177–179] have indeed implicated nociceptin in uterine pain. Our study is the first to localize nociceptin in uterine tissue and therefore leads a strong support to many physiological as well as pharmacological effects of nociceptin that have been reported in the literature.

The localization of nociceptin in the endometrium of uterus further supports the role of nociceptin as a putative modulator of the endocrine function in the endometrium. However, further studies are needed to establish the precise role of nociceptin in endometrial function. The other observation of our immunofluorescence study is the significant reduction in the uterine tissue level of nociceptin after the onset of DM. This observation corroborates our own findings on the pancreatic tissue level of nociceptin. However, it is impossible to compare our findings with that of the litrerature since this is the first study to examine the tissue level of nociceptin by immunofluorescence method in the uterus. Since some neuropeptides, such as galanin have a reduced pancreatic content after the induction of diabetes, it is not surprising to observe that the tissue level of nociceptin also decreases with DM [180].

Western blotting technique was performed to confirm the localization of nociceptin in uterine tissue. The findings of Western blot technique are in agreement with those performed with immunofluorescence.

In addition to the use of Western blot to confirm the presence of nociceptin in the uterus, we have also used immunoelectron microscopy to determine the exact location of nociceptin in the rat uterus. Our study showed that nociceptin is located on the myofibrils of the smooth muscle cells. This observation supports the role of nociceptin as a regulator of smooth muscle contraction. The number of gold particles on the myofibrils of non-diabetic control rat uterus was significantly higher compared to that of diabetic rats. This supports our findings with immunofluorescence as well as Western blot techniques.

5.3.1 Nociceptinergic innervation

The uterus is an organ which is associated with many structural and physiological changes with menstrual cycles, pregnancy and labor. It is likely that the uterine nerve supply relays sensory and motor information from and to the end organ during, and around, these physiological and or pathological changes. Afferent information conveyed from the uterus includes pain, distension and vascular data. Moreover, the female reproductive organs of humans and laboratory mammals receive well developed autonomic and sensory nerves containing different neurotransmitters such as noradrenaline (NA), acetylcholine (ACh) and several neuropeptides, such as neuropeptide Y (NPY), substance P (SP), vasoactive

intestinal polypeptide (VIP), neurotensin (NT), neurokinin A (SK), bombesin (BOM), calcitonin gene-related peptide (CGRP), cholecystokinin (CCK), galanin (GAL) and leucin-enkephalin [181–187].

The presence of nociceptin outside of the CNS has been observed where they are reported to perform non-neuronal roles. The present study demonstrates for the first time, the distribution pattern of nociceptin in the uterus of rats and supports the physiological and pharmacological role of nociceptin in the uterus. For example, nociceptin has been reported to induce uterine relaxation [86]. Gáspár et al. [188] reported that nociceptin regulates uterine contraction. The myometrium contains smooth muscles that are responsible for the contraction of the uterus. Many studies have shown that nociceptin regulates smooth muscle contraction [36, 57, 189–192]. While some studies have attributed a contractile role for nociceptin in smooth muscles, others have observed otherwise. In a further confusion, Yüce et al. [193] reported that nociceptin has no role in the contraction of smooth muscle. The density of nociceptin-containg nerves was significantly more in the myometrium compared to the endometrium.

However, the expression of nociceptin in the nerve of the myometrium of non-diabetic control compared to that of diabetic rats.

There are many reports showing that nociceptin inhibits the release of ACh [194–196]. For example, it has been shown that extracellular ACh is significantly higher in the hippocampus in NOP-receptor knockout mice. This indicates that the two bioactive agents may indeed be interrelated in function.

5.3.2 Conventional electron microscopy

Electron microscopy study was performed to investigate the ultrastructuarl basis of the atrophy of the uterine tissue observed using light microscopy. Ultrastructural study showed severe degeneration of cytoplasmic organelles including the cilia, myofibrils, nuclei of epithelial cells of the endometrium of diabetic rats compared to non-diabetic controls. In addition, a higher number of lipid droplets were observed in the epithelial cells of the endometrium of diabetic rats compared to controls. These observations support the light microscopy features of uterine atrophy reported in result's section. Uterine atrophy may pose a difficult problem for diabetic women trying to conceive and deliver a healthy offspring.

DOI:10.14753/SE.2016.1887

6. Conclusion

- The study showed that nociceptin is present and coexists with insulin in pancreatic β-cells.
- The degree of co-localization of nociceptin with insulin in pancreatic β-cell was altered in STZ-induced diabetes.
- The number of nociceptin labelled colloidal gold particles was significantly lower in the β-cell of the islet of Langerhans after the onset of diabetes compared to non-diabetic control.
- The study shows a gross atrophy of uteri as early as 15 days post DM.
- The study revealed the presence of nociceptin in the endometrium and myometrium of the rat uterus.
- The expression of nociceptin was significantly lower in diabetic rat uteri compared to those of non-diabetic control.
- The myometrium of both non-diabetic and diabetic rats contain nociceptin -positive nerves with varicosities.
- The expression of nociceptin in the nerve of the myometrium of non-diabetic control is higher compared to that of diabetic rats.
- Ultrastructural study showed that nociceptin is localized specifically to myofibrils of the smooth muscles of non-diabetic control and diabetic rat uteri.
- The degree of expression of nociceptin in myofibrils of the smooth muscles of diabetic rat uteri is signignificantly lower when compared to non-diabetic control.
- The outcome of this study will provide an insight into the physiology of nociceptin -mediated nociception in the uterus of normal and diabetic subjects and open ways to prevent uterine atrophy associated with DM.
- The localization of nociceptin in uterine wall suggests that it may have a specific physiological role in the modulation of uterine function.
- Additional knowledge on the role of nociceptin in the uterus may help in the development of nociceptin-based therapeutic drugs in the management of labour and other uterine conditions.

DOI:10.14753/SE.2016.1887

- The degeneration of uterine and loss of nociceptin, a neuropeptide implicate the regulation of pain and appears to be a “double edge sword” for female diabetics looking forward to getting pregnant.

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DOI:10.14753/SE.2016.1887

8. Publications

8.1 Publications related to the PhD thesis

1. **Tariq S**, Nurulain SM, Hameed RS, Lotfy M, Emerald S, Koturan S, Tekes K, Adeghate E. (2016) Diabetes-induced changes in the morphology and nociceptinergic innervation of the uterus. J Mol Histol, 47(1):21-33. IF: 1.815

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DOI:10.14753/SE.2016.1887

9. Summary

Nociceptin (NC) has been implicated in the pharmacology of the pancreas and the uterus with no information about the presence of NC in these organ systems. Different methods, including immunohistochemitry, immunofluorescence, conventional electron microscopy, single and double labelling immunoelectron microscopy and Western blot techniques were used to determine the localization and density of NC in the pancreas and uterus of non-diabetic and diabetic rats. The study also investigated whether NC co-localizes with insulin in the β-cells of pancreatic islet. The second part of our study examined the morphological changes associated with DM.

Our study showed that NC is present in pancreatic islet cells of both non-diabetic and diabetic rats. Quantitative analysis of NC-positive cells showed that the number of NC-positive cells was significantly ($p < 0.05$) lower in the islet of diabetic rats compared to non-diabetic controls. Immunofluorescence study also showed that NC co-localized with insulin in pancreatic β-cells. The degree of co-localization of NC with insulin was severely altered in diabetes. Moreover, immunolectron microscopy showed that either insulin- or NC-positive gold particles were observed on the secretory granules of β-cells. Morphometric analysis of NC-labelled gold particles showed, that it was markedly lower in diabetic rats when compared to that of non-diabetic controls.

The study showed a severe degeneration of cytoplasmic organelles of uterine cells as early as 15 days post DM which was confirmed by light and electron microscopy. The expression of NC in the cells and nerves of the endometrium and myometrium of diabetic rat uterus was significantly ($p<0.05$) lower compared to those of non-diabetic controls.

It can be concluded that NC is present in the endocrine pancreas and coexists with insulin in pancreatic β-cells. In addition, NC is discerned in the rat uterus. The expression of NC in pancreatic islet cells and in the uterus is significantly reduced after the onset of diabetes. This is the first morphological evidence of the co-localization of NC with insulin in pancreatic β-cells. This co-localization may play a role in pancreatic β-cell function. Our finding is also the first study demonstrating the localization of NC in the rat uterus. There is no available morphological evidence of this neuropeptide in the uterus of any animal species. The presence of NC in the uterine wall suggests physiological roles of NC in the modulation of uterine function.

DOI:10.14753/SE.2016.1887

9.1 Összefoglalás

A nociceptin (NC) szerepe a pancreas és az uterus működésében számos farmakológiai vizsgálatban bizonyítást nyert, bár a NC jelenlétét ezekben a szervekben nem vizsgálták. Immunhisztokémiai, immunfluoreszcens, hagyományos elektronmikroszkópos, egyszeres és többszörös jelölésű immunelektronmikroszkópos és Western blot módszereket alkalmaztunk a NC lokalizációjának és mennyiségének meghatározására kontroll és diabeteses patkányok pancreas és uterus szöveteiben. Vizsgálatainkban arra is választ kerestünk, hogy a NC együttes lokalizációt mutat-e az inzulinnal a pancreas β-sejtjeiben. Munkánk második részében azt vizsgáltuk, hogy milyen morfológiai változásokat okoz ezekben a szövetekben a diabetes.

Megállapítottuk, hogy a NC mind a kontroll mind a diabeteses patkányok pancreasának β-sejtjeiben kimutatható. A kvantitatív analízis azt mutatta, hogy a diabeteses patkányok NC-pozitiv sejtjeinek száma szignifikánsan ($p< 0.05$) alacsonyabb, mint a nem-diabeteses kontroll állatoké. Az immmunfluoreszcencia-vizsgálataink azt igazolták, hogy a NC és az inzulin ko-lokalizációt mutat a pancreas β-sejtjeiben. A NC és az inzulin ko-lokalizációjának mértéke jelentősen változott a diabeteses állatokban. Az immunelektronmikroszkópos vizsgálataink azt igazolták, hogy mind az inzulin-, mind a NC-pozitív arany-részecskék a pancreas β-sejtjeinek szekretórikus granuláiban helyezkednek el. A NC-jelzett arany-részecskék morfometriás analízise azt mutatta, hogy a diabeteses állatokban ezen sejtek száma szignifikánsan alacsonyabb, mint a nem-diabeteses kontrollokban.

Mind a fénymikroszkópos mind az elektronmikroszkópos vizsgálataink súlyos sejt-organellum degenerációt mutattak ki a diabeteses patkányok uterus-sejtjeiben már a diabetes fennállásának 15. napjától. A NC expressziója mind az endometrium sejtjeinek ideg-elemeiben, mind a myometrium sejtjeiben szignifikánsan ($p< 0.05$) alacsonyabb volt a diabeteses patkányok uterusában, mint a nem-diabeteses kontrollokéban.

Vizsgálataink során igazoltuk, hogy a nociceptin jelen van az endokrin pancreas β-sejteiben és ko-lokalizációban helyezkedik el az inzulinnal. Megállapítottuk továbbá, hogy a nociceptin jelen van az uterus szövetben és az alkalmazott módszereinkkel meghatározható. Megállapítottuk, hogy a nociceptin expressziója a pancreas β-sejtjeiben és az uterus sejtjeiben egyaránt szignifikánsan csökken diabetes esetén. Vizsgálataink az első morfológiai bizonyítékként szolgálnak arra vonatkozóan, hogy az inzulin és a nociceptin a pancreas β-sejtjeiben együtt helyezkedik el. Ez a ko-lokalizáció arra enged következtetni, hogy a nociceptinnek szerepe lehet a pancreas β-sejtjeinek működésében. Vizsgálataink a nociceptinnek a patkány uterus szövetben való jelenlétét és lokalizációjának meghatározását tekintve is az első kísérletes bizonyíték. Ezidáig a nociceptin, mint neuropeptid jelenlétét más állatfajban nem vizsgálták. A nociceptin jelenléte az uterus-fal sejtjeiben arra enged következtetni, hogy a nociceptinnek az uterus működésében élettani szerepe van.

DOI:10.14753/SE.2016.1887

10. Acknowledgements

I am extremely grateful to the Almighty God, who bestowed me strength and courage to complete this dissertation.

I express my deepest gratitude and unprecedented thanks to my supervisor, Professor Kornélia Tekes, Department of Pharmacodynamics, Semmelweis University for her expert guidance, continuous support throughout the course of my PhD program.

I would like to express my sincere thanks to Professor Ernest Adeghate, Department of Anatomy, CMHS, UAE University for his excellent expertise, keen interest and kind advice with support which enabled me to complete this task.

I am thankful to Professor Huba Kalász, Department of Pharmacology and Pharmacotherapy, Semmelweis University for his kind advice and sincere cooperation during the work.

My sincere thanks to Professor Safa Shehab, Director of Electron Microscopy and Imaging Unit, College of Medicine and Health Sciences, UAE University for providing necessary imaging facilities for the research work.

My gratitude extends to Dr Syed M Nurulain, Medical Research Specialist, Department of Pharmacology and Mr. R. S. Hameed, Medical Research Specialist, Department of Anatomy, CMHS, UAE University for their support and encouragement during the study.

My deep grateful and heartiest gratitude go to my dearest parents and beloved wife for their patience, encouragement and sincere cooperation throughout my studies but could not see the result and passed away before my thesis defense. I would like to dedicate my thesis to my beloved Parents and Wife who always prayed for my success.

Last but not the least; I would like to appreciate my children for their patience and innocent prayers for my success, which I am extremely obliged.

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11. List of tables and figures

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Figure 9 Immunofluorescence micrographs of nociceptin (red) and insulin (green) in the islets of non-diabetic (9a) and diabetic (9b) rats. Yellow colour indicates cells that contain both nociceptin and insulin. (9c): Morphometric analysis of insulin and nociceptin positive cells in non diabetic and diabetic rat pancreas.

Figure 10 non-diabetic rats (A) pancreatic β-cells showing a large number of secretory granules (SG), normal nuclear architecture (N), normal nuclear envelope (NE), normal endoplasmic reticulum (ER) and well developed mitochondria (M).

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Figure 18 Immunoflorescence micrographs of nociceptin-positive cells in the uterus of non-diabetic control (a) and diabetic (b) rat.

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Figure 20 Western blot analysis of nociceptin in the uterus of non-diabetic control and diabetic rats (A). Lane 1 shows a reduced nociceptin concentration in the uterus of diabetic rats compared to non-diabetic control (Lane 2).

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12. APPENDIX

Reagents and fixatives

Buffer for streptozotocin

1. 0.1 M Sodium citrate (molecular weight 294) 3.57g/100ml distilled water
2. 0.1 M Citric acid (molecular weight 210) 2.10g/100ml distilled water
3. Distilled water
4. Adjust the pH to 4.4-4.5

Take 2.2 ml of sodium citrate solution (1), add 2.8ml citric acid solution (2) and then add 5 ml of distilled water. Now dissolve streptozotocin @60mg/kg body weight in the buffer.

0.2 M phosphate buffer, pH 7.3 (PB)

21.8 g anhydrous sodium phosphate- dibasic

6.4 g anhydrous sodium phosphate -monobasic

1000 ml distilled water

Adjust pH 7.2-7.3

3.1.6 0.1M monobasic Phosphate Buffer)

1. KH2PO4 3.4g
2. NaH2PO4.H2O 10.4g
3. Distilled Water 1000 ml

   pH 7.2 (Add 36mls of 2M NaOH in final 1000mls)

Citrate buffer (pH 4.5) for immuno staining

5. 0.1 M Sodium citrate solution 220 ml
6. 0.1 M Citric acid solution 280 ml
7. Distilled water 500 ml
8. Adjust the pH to 4.5

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0.1 M Phosphate buffer saline (pH 7.4)

1. Distilled water 800 ml.
2. Sodium chloride 8 g
3. Potassium chloride 0.2 g
4. Sodium dihydrogen phosphate 1.44 g
5. Potassium dihydrogen phosphate 0.24 g
6. Add distilled water to a total volume of 1 liter.
7. Adjust the pH to 7.4

0.01 M phosphate buffer saline (PBS) for 1 liter:

0.2 M phosphate buffer = 50 ml
Na Cl = 9 g
Mi11i Q water = 950 ml
and adjust = pH 7.2

Zamboni's Fixative

0.2 M Phosphate Buffer – 1000 ml
Saturated Picric Acid – 300ml
Paraformaldehyde – 696 ml
Take 80 gms of paraformaldehyde in 696 ml of distilled water then warm the paraformaldehyde to dissolve for about 5-10 minutes. Let the solution cool, then add the phosphate buffer and saturated picric acid at pH 7.4

Karnovsky's fixative

0.2 M Phosphate buffer =50ml

25%Glutaraldehydes in water=10ml

10%Paraformaldehyde in ware=20ml

Add distilled water to make upto 100mls this fixative contain2.5 % glutqaraldehe and 2% para formaldehyde in 0.1M phosphate buffer and adjust pH at 7.2

McDowell and Trump Fixative (LR White) pH 7.2

1) 4% Paraformaldehyde

2) 0.1%Glutaraldehyde

3) 0.1M phosphate buffer

This gives a 4% paraformaldehyde and 0.1% glutaraldehyde solution which is suitable for a wide range of tissues and has rapid penetration. Store at 4°C in the fridge.

1% Osmium Tetroxide in 0.1M Phosphate Buffer

Add 2ml 4% OsO_4 in an ampoule to

8ml of 0.1 M Phosphate Buffer in a dark brown bottle

EPON Resin (Total = 51.00 gm)

1. Agar 23 g.
2. DDSA 14 g.
3. MNA 13 g.
4. DMP-30 1.1 g. Mix well for 5minutes to homogenize for use

Preparations of 0.2M Phosphate buffer and 4 % paraformaldehyde

a) 0.2 M phosphate buffer, pH 7.3 (PB)

21.8 g anhydrous sodium phosphate- dibasic

6.4 g anhydrous sodium phosphate -monobasic

1000 ml distilled water

Adjust pH 7.2-7.3

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b) 4% paraformaldehyde fixative:

Heat 500 ml Milli Q water in 1 liter beaker to 60°C. Do not exceed 65°C. Add 40 g paraformaldehyde, stir for several minutes and add a small squirt of 0.1 M NaOH (about 1 ml), keep stirring until solution is clear. Filter 500 ml 0.2 M phosphate buffer into same container as fix final solution is 4% paraformaldehyde in 0.1 M phosphate buffer.

Toluidine Blue Stain for semithin sections

a) 1% toluidine blue

b) 1%borax

c) warm distilled water 100 ml and mix well and filter for use

12% Uranyl Acetate stain for ultrathin sections

1- Uranyl acetate=12g

2- Distilled water=100ml

Lead citrate

1-Lead nitrate=1.33g in15ml d/w

2-Sodium citrate=1.77g in 15 ml d/w

Dissolve separately and Mixed vigorously to make it clear then add 8 ml 1N NaOH and then add 12 ml D/W to make 50 ml stain and then filter and centrifuge foe use as a stain.