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EFFECTS OF NOCICEPTIN ON MARKERS OF OXIDATIVE STRESS AND METABOLIC PROFILES IN DIABETES MELLITUS

PhD Dissertation

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2. List of Abbreviations

AA	Amino Acid
CAT	Catalase
CNS	Central nervous system
DAG	Diacylglycerol
DM	Diabetes mellitus
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
FFA	Free fatty acid
GDM	Gestational Diabetes Mellitus
GPCRs	G-protein coupled receptors
GIT	Gastrointestinal tract
GLUT2	Glucose transporter 2
GRED	Glutathione reductase
HG	Hyperglycemia
IF	Immunofluorescence
i.p.	Intraperitoneal
IDDM	Insulin-dependent diabetes mellitus
IEM	Immuno electron microscopy
INS	Insulin
KB	Krebs buffered
LRW	London resin white
N/OFQ	Nociceptin/OrphaninFQ
NC	Nociceptin
NIDDM	Non insulin-dependent diabetes mellitus
NOP	Nociceptin opioid peptide
OFQ	Orphanin FQ
PBS	Phosphate buffered saline

PP	Pancreatic polypeptide
RIA	Radioimmunoassay
ROS	Reactive oxygen species
SOD	Superoxide dismutase
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TEM	Transmission Electron microscopy

3. Introduction

3.1. Nociceptin

Nociceptin (NC) is a small (17-amino acids) endogenous peptide located in the central nervous system [1]. NC, commonly referred to as orphanin FQ (N/OFQ), was discovered in 1995 by two groups of scientists (Meunier et al.; Reinscheid et al.) working independently of each other. NC was originally called „orphanin" because it was deemed to be a peptide of an orphan receptor, which was discovered way before the ligand. This was indeed, a classical example of reverse pharmacology [2, 3]. NC is a product of a much larger precursor protein, prepronociceptin, which is located on chromosome 8, at p21.1 (Fig. 1.).

Fig. 1. Amino acid (AA) sequence and chromosomal location of nociceptin

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

Chromosomal Location: Chromosome 8, p21.1

Modified from Tariq et al [44]

Since its discovery in 1995, NC has attracted the interest of many scientists. A total of 2,080 articles were indexed in PubMed as of May 2, 2022 [4].

The nociceptin (NC) ligand acts on nociceptin opioid peptide (NOP) receptor. This receptor is also called nociceptin/orphanin FQ- (N/OFQ) or k-type 3 opioid receptor. It is encoded by the opioid receptor-like 1 gene [5]. NOP belongs to one the largest family of receptors in the human body, the G protein-coupled receptors (GPCRs). These receptors in association with their ligands play important roles in the function of the brain and other parts of the central nervous system [6]. The NOP receptor is a large protein consisting of 370 amino aids (aa) in humans. It has seven transmembrane units, where the N-terminal has 44 aa with three additional areas (Asn-X-Ser/Thr) for glycosylation [5]. Moreover, the 3rd and the 2nd intracellular loops of NC can be phosphorylated by protein kinase A and C, respectively [5, 6]. Using [11C]NOP-1A

positron emission tomography, at least 3 isoforms of NOP have been identified in man [7]. Even though the different isoforms of NOP shares up to 60% similarity with classical opioid receptors such as μ-OP, κ-OP, and δ-OP, it has no significant affinity for classical opioid molecules like morphine and true the other way round [8].

A large variety of ligands that act as either agonists (in addition to nociceptin) or antagonists have been identified. Currently named ligands acting as agonists for NOP receptors including the following: Buprenorphine (partial agonist), BU08028 Cebranopadol (full agonist at NOP), Etorphine, MCOPPB [35] (full agonist) Norbuprenorphine (full agonist). The following ligands on the other hand AT-076 (non-selective), JTC-801, J-113,397 SB-612,111, SR-16430, and Thienorphine are classified as antagonists [9-10].

3.1.1. Tissue distribution of nociceptinergic system

The pattern of distribution of NC and its receptors in the central nervous system has been well characterized. NC has been shown to be localized to different regions of the central nervous system (CNS) including but not limited to the hypothalamus, arcuate nucleus and tegmentum of the midbrain [11-12]. Large amount of NC-, and NOP-positive neurons and nerve profiles are extensively located in the cortical regions of the brain, olfactory structures, hippocampal, amygdaloid and thalamic regions. NC and NOP receptor-containing nerve elements have also been observed in different parts of the brain stem and in the grey matter of the spinal cord [13-17]. NC and NOP receptors have been detected in the gastrointestinal tract, ductus deferens, and in the cells of the immune system [18-20]. The detection of NC, NOP receptors and their mRNAs in CNS and peripheral nervous systems and other non-neural organs show that the nociceptinergic system is ubiquitous to the human body. It also shows why the nociceptinergic system is involved in a large variety of physiological and pathological processes (Table 1).

Table 1 Tissue distribution of the nociceptinergic system

Organ/Body System	Structure/Cell	Ref #
CNS	hypothalamus, arcuate nucleus, and tegmentum of the midbrain, cortex of brain, olfactory bulb, hippocampus, amygdaloid and thalamic nuclei.	[11-17].
GIT	Intestine	[18]
Reproductive system	Ductus deferens	[19]
Immune system	Immune cells	[20]
Endocrine system	Islet of Langerhans	[21]

3.1.2. Physiological effects of nociceptinergic system

The discovery of the ligand (NC) for NOP receptors have indeed opened the way for investigating the role of this system in different physiological and pathological mechanisms. It also encouraged researchers to find antagonists and agonists for the NOP receptor. The mechanism by which the nociceptinergic system exerts its function has not been completely elucidated but it has been shown that when NOP receptor is activated it inhibits the activation of adenylyl cyclase and Ca^{2+} channels while stimulating K^{+} channels in a process similar to that observed in the case of opioids. In this way, NOP receptors inhibit the sympathetic nervous system causing a decrease in mean arterial pressure and respiration rate [22]. It has been reported that the nociceptinergic system can modulate nociception [23], musculoskeletal function [24], inhibition of pain [25], lowering of the stimuli of stress [26], memory and acquisition of cognition [27], neurotransmitter and hormone release [28, 29], kidney function [30], differentiation of neurons [31], sexual and reproductive behavior [32], pruritus [33], contractions of myometrium [34], food intake [35], traumatic stress [36] anxiety [37], motility of the gastrointestinal tract [38], cardiac and vascular functions [39], urination [40], and reflex of tussis [41]. Moreover, the nociceptinergic system has been shown to play a role in the modulation of hypoxic-ischemic induced brain lesion [42],

thermoregulation [43], vestibular function [44] and many others. The roles of the nociceptinergic system in different physiological functions has been previously described by Tariq et al. [45]

NC has also been shown been implicated in a large variety of pathological diseases. The plasma level of NC is increased in postnatal and major depressions, Wilson's disease, hepatocellular cancer, pain (both acute and chronic), sepsis, biliary cirrhosis, ischemic attack of the transient type, stroke, angina pectoris and bipolar disorder [45]. In contrast, the plasma level of NC is significantly reduced in fibromyalgia syndrome, headache (of the cluster type), migraine, limb and cardiac ischemia and atherosclerosis [45]. It was interesting to note that the level of NC does not change in patients with diabetic neuropathy [46].

3.2. Diabetes mellitus

Diabetes mellitus (DM) is a common metabolic disorder affecting millions of people across the globe. Currently, more than 537 million people live with DM [47]. It is also projected that the prevalence of DM will continue to rise, reaching an astronomically high number of 783 million by 2045 [47]. DM is characterized by hyperglycemia because of insufficient or ineffective insulin molecule to help in the uptake of glucose molecules by target cells such as skeletal muscle, hepatic and fat cells [48]. The inability of the cells to use glucose leads to disruption in the metabolism of carbohydrates, lipids and proteins [49]. Hyperglycemia-induced oxidative stress, coupled with the disruption in the metabolism of carbohydrates, lipids and proteins leads to chronic complications of DM such as diabetic- retinopathy, cardiomyopathy, nephropathy, neuropathy, macro- and micro-angiopathy. These chronic complications of DM subject the patient to severe morbidity and eventually mortality [50]. The common symptoms of DM include but are not limited to increased thirst and urination; fatigue, nausea and vomiting; urinary bladder infection, candidiasis and poor visual acuity.

3.2.1. Types of diabetes mellitus

The more recent classification puts DM into three main categories, T1DM, previously known as insulin-dependent diabetes mellitus (IDDM) or juvenile diabetes, T2DM, previously referred to non insulin-dependent diabetes mellitus (NIDDM) and lastly Gestational diabetes (GDM), which has its onset at the time of pregnancy.

3.2.1.1. Type 1 diabetes mellitus

T1DM makes about 10% of all people with DM and results from failure of the pancreas to secrete sufficient insulin. T1DM is characterized by severe loss or the necrosis of pancreatic beta cells, the cells responsible for the production of insulin, resulting in little or no insulin and hyperglycemia [50]. This type of DM is very common in the young and adolescent. It is caused by pancreatic beta cell destruction either by autoimmune cells, viral infection or toxic agents [51].

3.2.1.2 Type 2 diabetes mellitus

T2DM comprises of about 90% of all DM cases and is more prevalent in the adult population. T2DM is characterized by the inability of the insulin molecule to effectively help in the uptake of the glucose molecules to cells such as skeletal muscle, liver and fat cells. This phenomenon is referred to as insulin resistance. It has been suggested that one of the main cause of insulin resistance is defective insulin receptor [52]. T2DM is the most common form of DM and it is commonly associated with the metabolic syndrome of obesity, DM and hyperlipidemia [50].

3.2.1.3 Gestational diabetes

GDM occurs when pregnant women without a previous diagnosis of diabetes develop chronic hyperglycemia, which may eventually lead to overt DM. It may precede development of type 2 DM. GDM develops in the background of insulin resistance or reduced insulin secretion observed during mid-pregnancy and persists through the third trimester until the baby is delivered [53]. GDM has been referred to as „just" an abnormally high blood glucose level and not necessarily a disease [54]. GDM is

observed in about 5–10% of all pregnancies, but its prevalence varies in from place to place, depending on racial background, genetic and environmental factors [55]. The risk factors for GDM include but not limited to polycystic ovary syndrome, tobacco consumption, family history of DM, and older age.

3.3. Diabetes mellitus and oxidative stress

Reactive oxygen species (ROS) include radical (superoxide anion, hydroxyl radical, alkoxyl radical, peroxyl radical) and non-radical (hydrogen peroxide, singlet oxygen, hypochlorous acids) species. Reactive nitrogen species (RNS) on the other hand contain nitrogen-centered species. They include nitric oxide and peroxynitrite [56]. It has been shown that chronic hyperglycemia (HG) can lead to the release of ROS and RNS through the activation of both enzymatic and non-enzymatic pathways. One of such pathways in the polyol pathway in which aldose reductase is increased leading to elevated sorbitol level in tissues. Increased sorbitol level induces cellular osmotic pressure and a reduction in endogenous antioxidant levels leading oxidative stress and tissue damage [57].

In addition, chronic HG increases the activity of the hexosamine pathway leading to excessive release of uridine diphosphate-N-acetyl glucosamine, a molecule that cause elevation of tissue levels of transforming growth factor-beta1 (TGF-beta1). TGF-beta 1 contributes to the development of diabetic angiopathy [57].

Moreover, long-term elevation of blood glucose also stimulates protein kinase-C pathway via increases in the level of diacylglycerol (DAG). DAG is a known stimulant of vascular endothelial growth factor, methylglyoxal and other proteins that are capable of inducing vascular porosity, tissue hypoxia and oxidative stress. Other pathway in which chronic HG induces oxidative stress is via increased production of Advanced Glycation-end (AGE) Products. Accumulation of AGE products leads to oxidative and impaired cellular functions [57].

3.4. Markers of oxidative stress

Markers of oxidative stress can be classified into those that examin Measuring the net antioxidant capacity of the serum lipid peroxidation (Isoprostanes, malondialdehyde, oxidative protein modifications (nitrotyrosine, S-glutathionylation), myeloperoxidase, ROS-induced modification in genes (Nrf-2) and determination of endogenous antioxidants [58].

In this study we have examined the level of key endogenous antioxidants including CAT, SOD and GRED.

Catalase (CAT) is a ubiquitous enzyme capable of converting millions of molecules of harmful hydrogen peroxide into oxygen and water, thereby reducing oxidative stress in cells and tissues [59]. Superoxide dismutase (SOD) neutralizes the superoxide radical (a ROS), turning it into oxygen and hydrogen peroxide. The hydrogen peroxide produced in then destroyed by CAT [60]. Glutathione reductase (GRED) which is conserved in all living organisms contribute to the formation of glutathione, an molecule critical to the reduction of oxidative stress. GRED together with glutathione protect cells from oxidative stress by converting hydrogen peroxide to H_2O_2 and peroxide. GRED is also capable of neutralizing ROS such as hydroxyl radicals and singlet oxygen [62].

3.5. The role of peptides in DM

Many peptides including ghrelin, galanin, resistin, calcitonin-gene-related peptide and many others have been observed in insulin-producing beta cells of the endocrine pancreas. This observation suggests that peptides, including islet peptides do have a role in the pathogenesis of DM [49].

3.6. The role of the nociceptinergic system in the etiopathogenesis of DM

Literature reports have indicated that NC may play a role in the modulation of pain associated with diabetic neuropathy, a common chronic complication of DM. Indeed, when administered intrathecally, NC significantly reduced the pain associated with DM [62]. Moreover, NC has been localized to the insulin-producing pancreatic beta cells but its role on insulin metabolism is not clear.

4. Objectives

4.1. Hypothesis

We hypothesize that nociceptin (NC) can ameliorate the signs and symptoms of diabetes mellitus in addition to increasing the expression of endogenous antioxidants in several organ systems.

4.2. Aims and objectives

Our aim in this project is to investigate the tissue and cellular localization of NC in the pancreas. We also wanted to determine whether nociceptin can influence endocrine release from the islet of Langerhans of both normal and diabetic rats. Since DM rats suffer from oxidative stress, we wanted to know if NC can increase the expression of endogenous antioxidants.

The following objectives were addressed:

a. **To determine tissue and cellular localization of NC**
b. **To examine the effect of NC on endocrine release in normal and diabetic rats**
c. **To investigate the effect of NC on the weight and glucose level**
d. **To determine whether NC can affect the expression of endogenous antioxidants such as catalase, glutathione reductase and superoxide dismutase in the kidney, liver and brain of normal and diabetic rats**
e. **To determine the effect of NC on endogenous antioxidant (catalase, glutathione reductase and superoxide dismutase) expression in pancreatic islet cells**

5. Methods

5.1. Experimental animals used in this study

The animals used in this study were Male Wistar rats bred at the Animal House of the College of Medicine & Health Sciences, United Arab Emirates University, Al Ain, UAE. The original strain was bought from Harlan Laboratories, Oxon, England, UK. All experimental animals were placed in large plastic (polypropylene) cages manufactured specifically for murine models. The Animal Facility was fully air-conditioned (23 °C) with 12 h day and 12 h night cycle. Drinking water and rodent laboratory feed obtained from Emirates Feed Factory, Abu Dhabi, United Arab Emirates, were available *ad libitum*.

5.2. Diabetes induction

Diabetes mellitus was induced in male 150 - 200 g Wistar rats by a single dose of streptozotocin (STZ) [(60 mg/kg^{-1} body weight; Sigma, Poole, UK, given intraperitoneally, (i.p.)]. STZ was prepared in a buffered citric acid solution. The solution contains, 0.1 M citric acid and 0.1 M sodium citrate. The pH of the buffered solution was adjusted to 4.5. The same volume (0.3 ml) of this vehicle (buffered citric acid) solution was given i.p. to control rats. The confirmation of DM was performed using a One Touch II Glucometer (Life Scan Inc., Johnson & Johnson, Chesterbrook, PA, USA). Rats were considered diabetic if the fasting blood glucose level ≥ 10 mM (180 mg/dl). Experimental rats were euthanized one month after the induction of DM. Ethical approval for the study was obtained from the CMHS Animal Research Ethics Committee (A5-14).

5.3. Treatment of experimental animals with NC

NC was purchased from Abcam (Cat #: ab38198; aa1-17). NC was dissolved in phosphate buffered physiological saline (PBS) and given i.p. to rats at a dose of 10 μg/ kg per day for a total of five days to non-diabetic control and diabetic rats. The drug (NC) was administered at 9:00 every morning. Equal amounts of vehicle (PBS) were

administered i.p. to another group of 6 non-diabetic rats (normal control) and diabetic rats (diabetic controls) for the same experimental duration as treated. In order to allow the total excretion of STZ from the body of diabetic rats, NC and the vehicle were only given 15 days after the induction of DM.

5.4. Collection of blood and tissue samples

5.4.1. Immuno-localization of NC in the islet of Langerhans

In the experiment for the localization of NC in the serum and pancreatic islets, blood and tissue samples were collected four weeks post-induction of DM. Rats were euthanized and the pancreas was quickly removed *in toto*. The whole pancreas was portioned into for three separates studies: i). Immunofluorescence (IF) study, ii). Transmission electron microscopy (TEM) and, iii). Insulin secretion.

5.4.2. Effect of NC on markers of oxidative stress

Five days after treatment with NC, blood was collected from the inferior vena cava. In addition, the pancreas, kidney, liver and brain were harvested to study the effect of some selective markers of oxidative stress.

5.5. Fixation of pancreatic tissue fragments

Pancreatic tissue fragments were fixed overnight in Zamboni's solution for immunofluorescence study according to a previously reported method [63]. Small (2 mm^3) fragments from the body of the pancreas were fixed overnight in McDowell solution [64] for electron microscopy.

5.6. Immunofluorescence of pancreatic islets of Langerhans

Pancreatic tissue fragments taken from non-diabetic (n = 6) and diabetic (n = 6) rats were fixed in Zamboni's fixative [63] for 24 h at 4 °C. The pancreatic tissue fragments were then placed in ascending concentrations of ethyl alcohol, embedded in paraffin, and processed for immunofluorescence according to previously described technique [65]. 6 μm thick sections were cut using a Shandon AS325 microtome (Kalamazoo, MI, USA). In brief, NC was immunolabelled with TRITC (Jackson ImmunoResearch Inc.,

Westgrove, PA, USA) after incubation of deparaffinized sections with a blocking agent (UltraCruz® Blocking Reagent; sc-516214) and primary polyclonal antibodies against NC (rabbit nociceptin (1:100, Santa Cruz; R-20) (Table 2). The sections were then incubated for 24 h at 4 °C with monoclonal immunoglobulins raised against either insulin, glucagon, somatostatin, or PP (pancreatic polypeptide) (Dako, Glostrup, Denmark). Primary antibodies against either insulin, glucagon, somatostatin, or PP were immunolabelled with FITC (Jackson ImmunoResearch Inc.). The immunolabelled sections were mounted with Immunomount® (Shandon Inc., Pittsburgh, USA) and examined with a Zeiss fluorescent microscope (Carl-Zeiss-Strasse 22, 73447 Oberkochen, Germany).

Table 2 Dilution of the 1° and 2° antibodies

1° antibody	**Dilution**	**2° antibody**	**Dilution**
NC (Santa Cruz, CA, USA)	1:100	TRITC (Jackson ImmunoResearch Inc., Westgrove, PA, USA)	1:100
Insulin (Dako, Glostrup, Denmark)	1:1000	FITC (Jackson)	1:100
Glucagon (Dako)	1:1000	FITC (Jackson)	1:100
Somatostatin (Dako)	1:1000	FITC (Jackson)	1:100
PP (Dako)	1:1000	FITC (Jackson)	1:100

5.7. Immunoelectron microscopy study

Small fragments of pancreatic tissue were cut into small ($1mm^3$) pieces, fixed in McDowell's solution at 4 °C for 24 h and processed for IEM (immunoelectron microscopy) according to a previous technique [65]. In brief, pancreatic tissue samples were post-fixed in 1% OSO_4, dehydrated in graded concentrations of ethyl alcohol before embedding in LRW. One μm thick semithin sections were made. The areas of interest were selected for ultrathin sections. Ultrathin sections were made and placed on nickel grids. These grids were then processed for IEM, before review by Philips TEM.

5.8. Stimulation of rat pancreas (normal and diabetic) with NC

Normal and diabetic rat pancreas were expeditiously removed from anesthetized animals, cut into small pieces (1.0 mm^3) before incubation in Krebs buffered (KB) solution (contents in mM: NaCl, 118; KCl, 4.5; KH_2PO_4, 1.4; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 2.8) for 1 h. The pH of the buffered solution was 7.4. Incubation of the tissue in different concentrations of NC (10^{-6}, 10^{-9}, 10^{-12} M) was performed at 37 °C in a water bath and constantly aerated with physiological gas mixture (95% O_2 + 5% CO_2) in accordance to a previous technique [65]. The basal control contained only KB. The dose chosen has been shown to be effective in inhibiting vascular dilatation in the brain [66]. At the end of NC-induced stimulation of pancreatic tissues, the tissue samples were dried, weighed and the supernatant was retrieved. The supernatant was kept at -20 °C for insulin RIA and glucagon ELISA.

5.9. Insulin RIA

Radioimmunoassay method was used to measure the amount of insulin released into the supernatant after stimulation with different concentrations of NC using a sensitive rat insulin kit (Sigma-Aldrich catalog #: SRI13K). The measurement was performed according to the manual of instruction provided with the kit. All tests including the samples and controls were done as duplicates. In brief, 100 µl of calibrating reagents and test samples were placed in labelled tubes, in addition to 1 ml of 125I-insulin and vortexed before overnight incubation at 4 °C. One ml of precipitating reagent was then added, vortexed and transferred for 3 min prior to radioactivity counting with a gamma counter machine (Beckman, Fullerton, CA, USA). The lowest rat insulin concentration that could be measured with this method was 0.02 ng ml^{-1}. The data obtained were analyzed using Beckman Immunofit EIA/RIA analysis software (Version 2.00) and expressed in ng/ml/100 mg tissue of the pancreas.

5.10. Determination of glucagon concentration

After the stimulation with NC (10^{-6}, 10^{-9}, 10^{-12} M) the concentration of glucagon level in the supernatant was determined using ELISA kit (Catalog #. EK-028-02; Phoenix

Pharmaceuticals, Burlingame, CA, USA). The measurement of the concentration of glucagon was conducted based on the instructions given in the instruction manual. Test samples and controls were processed in duplicates. The readings were presented as ng/ml/100 mg wet tissue of the pancreas.

5.11. Pancreatic islet isolation and stimulation with NC

Islets of Langerhans were isolated from normal (non-diabetic) and diabetic rats using a previously established technique [67]. In brief, collagenase (Collagenase-P, #11249 002001; Merck, Darmstadt, Germany) solution (15 mg/15 ml) was administered directly into the pancreas via the common bile duct. The distended pancreases were put into flasks and incubated at 37 °C in a water bath, filtered through a 400 μm mesh tissue. The islets thus obtained were placed in Histopaque solution (Merck, Darmstadt, Germany) and the collected into Krebs buffered solution containing 2.8 mM of glucose. The islets were incubated with different concentrations of NC (10^{-6}, 10^{-9}, 10^{-12} M). The control group of islets was suspended in KB solution only. After incubation of islets with NC, the supernatant was decanted and kept at -80˚C until processed for insulin ELISA kit. Pancreatic islet mass was quantified with Countess II (Thermo Fisher, Waltham, MA, USA).

5.12. Measurement of insulin release from the islets of Langerhans

After NC stimulation of pancreatic islet cells, the insulin released was measured in the supernatant using ultrasensitive rat insulin ELISA kit (Catalog #: 10-1251-01; Mercodia Sylveniusgatan 8A, Uppsala, Sweden). Insulin level was done according to the protocol provided by vendor. All samples and calibrating controls were run in duplicates. Insulin level was given as μg/L/100 islets.

5.13. Morphometric analysis of endocrine cells

After immunohistochemical staining of the islets of Langerhans of non-diabetic (control) and diabetic rats, the number of nociceptin-, insulin-, glucagon-, somatostatin- and PP-positive cells was counted with Image J® (NIH, Bethesda, Maryland, USA).

The number of either nociceptin-, insulin-, glucagon-, somatostatin-, or PP-immunoreactive cells were tabulated and divided by the islet's total number of cells to determine the percentage of each type of cells in a given islet of Langerhans. A total of 5 islets were counted for each animal per group of six animals (n = 6).

5.14. Estimation of the number of NC- and insulin-containing secretory granules

In order to quantify the extent of the distribution of NC and insulin in pancreatic beta cells, the number of secretory granules containing either insulin, NC or both (5 nm, insulin; 10 nm, NC) were estimated on EM images. 5-6 images were counted for each animal per a group of six.

5.15. Immuno-expression of endogenous antioxidants in parenchymal organs

As one aspect of our study was to examine on the effect of NC on selected endogenous antioxidants, we used 6 μm thick sections for the immunochemical localization of these bioactive agents. Six μm thick sections mounted on gelatin-coated glass slides were taken through double-labelled immunofluorescence process to identify the pattern of distribution of endogenous antioxidants in the kidney, liver, pancreas and the brain in accordance with a published technique [65]. Briefly, deparaffinized and hydrated tissue sections were treated with citrate buffered solution before overnight incubation in the 1° antibody against catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GRED) followed by a 24 h treatment with the 2° antibody (Table 1). All incubations were performed 4°C unless otherwise specified. After incubation in conjugated TRITC, the tissue sections were rinsed in PBS and mounted on glass slides with CITI-Fluore medium (Science Services GmbH, Munich, Germany).

5.16. Serum catalase activity

The activity of catalase in serum of all animal groups (normal, normal treated, diabetic untreated, and diabetic treated) following the administration of NC was measured using a colorimetric method supplied with commercial kits obtained from Cayman Chemicals (Ann Arbor, MI, USA).

5.17. Immunofluorescence images

All fluorescence images in both pancreatic islets and other organs (kidney, liver, brain) were obtained with an AxioCam HRc digital camera using AxioVision 3.0 Software (Carl Zeiss, Oberkochen, Germany). Images were processed using Image J 1.8. The fluorescence images were taken without taking into account the intensity (density) of immunofluorescence.

5.18. Density of immunofluorescence in kidney, liver and brain

After the normal and diabetic rats were treated with NC, the density of the immunofluorescence staining of CAT, SOD and GRED in the renal cortex, liver, cerebral cortex and C3 region of the hippocampus was determined with Image J software® (NIH, Bethesda, MA, USA). The density of immunofluorescence is directly proportional to the cellular levels of these endogenous antioxidants in the kidney, liver and brain. Since the objective of the study is to examine whether CAT, SOD and GRED co-localize with islet hormones, the density of immunofluorescence in pancreatic sections was not measured. In brief, immunofluorescence images were posted on clipboard, inserted on 8-bit slot of Image J. After the conversion of the image, the total number of pixels was recorded using the line tool. The maximum (peaks) pixel were later taken as percentages of the control. The control (normal) image was taken as 100% . Data were measured as mean ± SEM (n=6 per group).

5.19. Chemicals and immunochemical reagents

All chemicals, reagents and immunochemical reagents were purchased from Merck (Darmstadt, Germany) unless otherwise stated.

5.20. Statistical Analysis

All experimental data were calculated as mean ± standard error of the mean. Differences between the groups were calculated using One-way ANOVA. Significant differences between mean values of the group, and two different timelines, were calculated with an unpaired t-test. Statistical significance was set at a value of $p < 0.05$. The post hoc test was analyzed using the Bonferroni test where the t- test was divided by the number of

groups being tested. 4 groups were used. $\alpha/n = 0.05/4 = 0.0125$.

6. Results

6.1. Total body weight and concentration of blood glucose

The induction of DM caused a significant ($p < 0.002$) loss in body weight when compared to non-diabetic controls 4 weeks into DM. In addition, rats suffering from DM appeared frail with polyuria and polydipsia compared to non-diabetic rats. Some animals developed cataract. Blood glucose concentration was markedly ($p < 0.0001$) higher in diabetic rat, when compared to control. (Table 3).

Table 3 Weight and blood glucose in normal (non-diabetic) and diabetic rats

	Non-diabetic rat	Diabetic rat
Weight (g)	330.5 ± 53.3 (n=6)	200.8 ± 47.2 (n=6)*
Fasting plasma glucose (mg/dl)	97.0 ± 5.2 (n=6)	411.8± 29.6 (n=6)**

*$p < 0.002$ vs control; **$p < 0.0001$

6.2. Pattern of distribution of NC in pancreatic islet cells of non-diabetic and diabetic rats

6.2.1. NC and insulin in islet cells

We wanted to know whether NC co-localizes with insulin in pancreatic islet cells and to determine whether diabetes alters the pattern of distribution of NC in the islet of Langerhans. Four weeks after the onset of DM, the number of NC-immunoreactive cells in the islets of Langerhans of diabetic rats was significantly reduced compared to control.

Using double labeling immunofluorescence, we observed that NC co-localizes with insulin in pancreatic beta cell both normal and diabetic rats. DM caused a marked ($p < 0.0001$) decrease in the number of insulin-positive cells compared to non-diabetic rats (Fig. 2.). The extent of co-localization is very high because the percentage of NC- and insulin-immunoreactive cells was similar in both non-diabetic and diabetic rats (Fig. 2.).

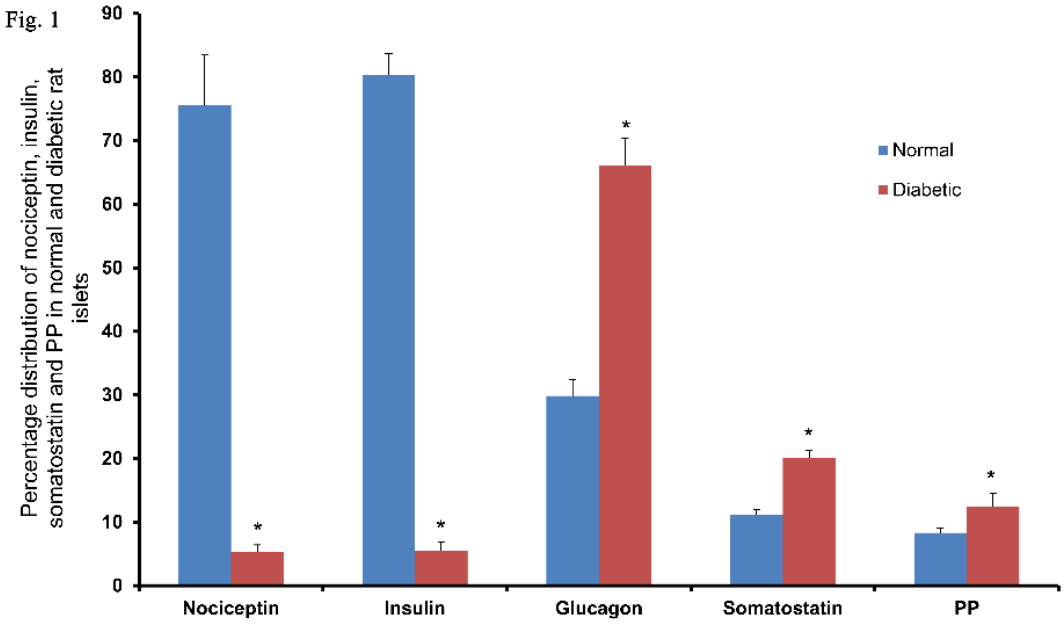


Fig. 2. Distribution of nociceptin- (NC), insulin-, glucagon-, somatostatin- and pancreatic polypeptide (PP)-immunoreactive endocrine cells in the islets of Langerhans of normal and diabetic rats. The percentage distribution of NC, insulin, glucagon, somatostatin and PP cells was markedly ($p < 0.05$) reduced after the induction of DM. Note the similarity in the pattern of distribution of NC and insulin. $n = 6$. *$p < 0.05$

*(From Adeghate et al., Cell & Tissue Research **374**:517–529, 2018)*

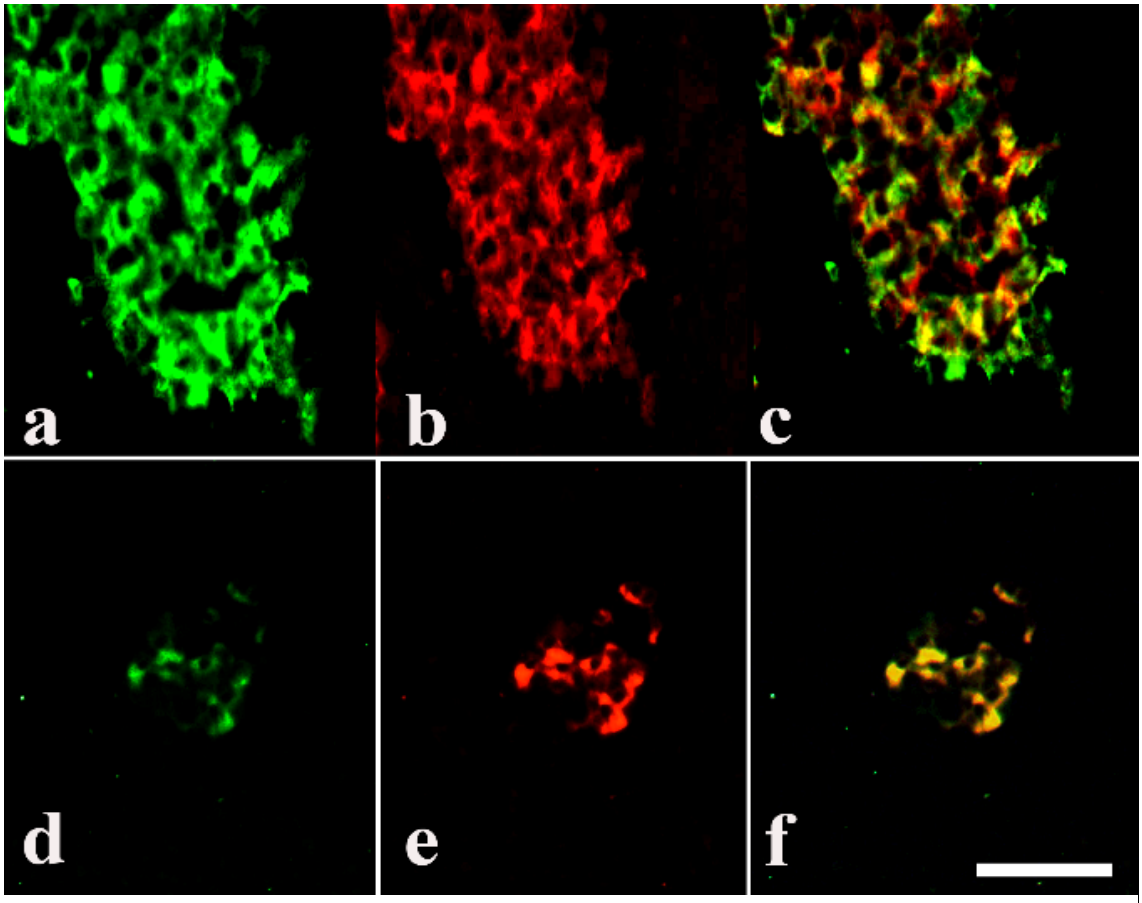


Fig. 3. Fluorescence images showing **(a)** insulin- and **(b)** NC-immunopositive cells in the islets of Langerhans of control rats. Immuno-expression of insulin **(d)** and NC **(e)** in the islets of diabetic rat is markedly reduced. Insulin co-localizes with NC **(c** and **f** shows merged images of insulin (green) and NC (red). The co-localization is depicted in yellow). n = 6. Scale bar = 50 μm

*(From Adeghate et al., Cell & Tissue Research **374**:517–529, 2018)*

6.2.2. NC and glucagon in pancreatic islets

Since it was not sure whether NC co-localizes with glucagon, we used double labeling immunofluorescence to determine their localization in pancreatic islet cells. Glucagon was observed mainly in the periphery of the islets of Langerhans while NC was mainly located in the central portion of islets (Fig. 3.). Double labelled IF showed that NC does not co-localize with glucagon in the alpha cells of the islets of Langerhans. It is worth noting that DM alters the pattern of distribution of glucagon-positive cells. In DM,

glucagon-positive cells increase markedly ($p < 0.0001$) in number, occupying both the peripheral and central portions of the islets of Langerhans (Fig. 4.).

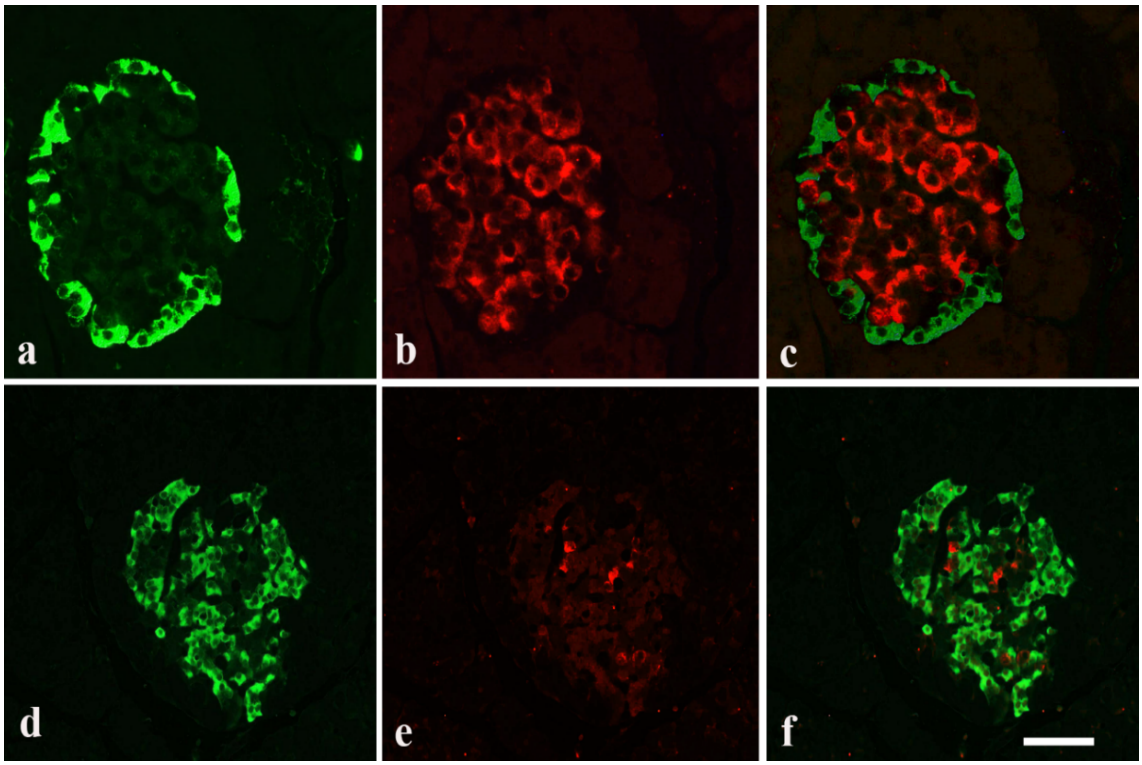


Fig. 4. Immunofluorescence images of **(a)** glucagon- and **(b)** NC-positive cells in pancreatic islets of control rats. Immuno-expression of glucagon **(d)** is elevated while that of NC **(e)** is reduced markedly in diabetic rat islets. No co-localization was observed between glucagon and NC [**(c** and **f** are merged images of glucagon (green) and NC (red)]. n = 6. Scale bar = 50 µm. *(From Adeghate et al., Cell & Tissue Research **374**:517–529, 2018)*

6.2.3. NC and somatostatin in islet cells

Somatostatin-immuno-positive cells are located in the peripheral region of pancreatic islet. NC on the other hand is seen in the central portion of islets of Langerhans. Double labelled IF was performed to determine if there is any co-localization between these two hormones in the delta cells of pancreatic islets. Double labelled IF showed that somatostatin and NC do not co-localize within any cells of the islet of Langerhans. However, DM alters the pattern of distribution of somatostatin-immunoreactive cells in

pancreatic islets. In DM rats, somatostatin can be observed in the central region of the islets instead of the peripheral location in normal rats (Fig. 5.). Moreover, the percentage number of somatostatin-positive cells is markedly ($p < 0.0001$) higher in the islet of DM rats (Fig. 5.).

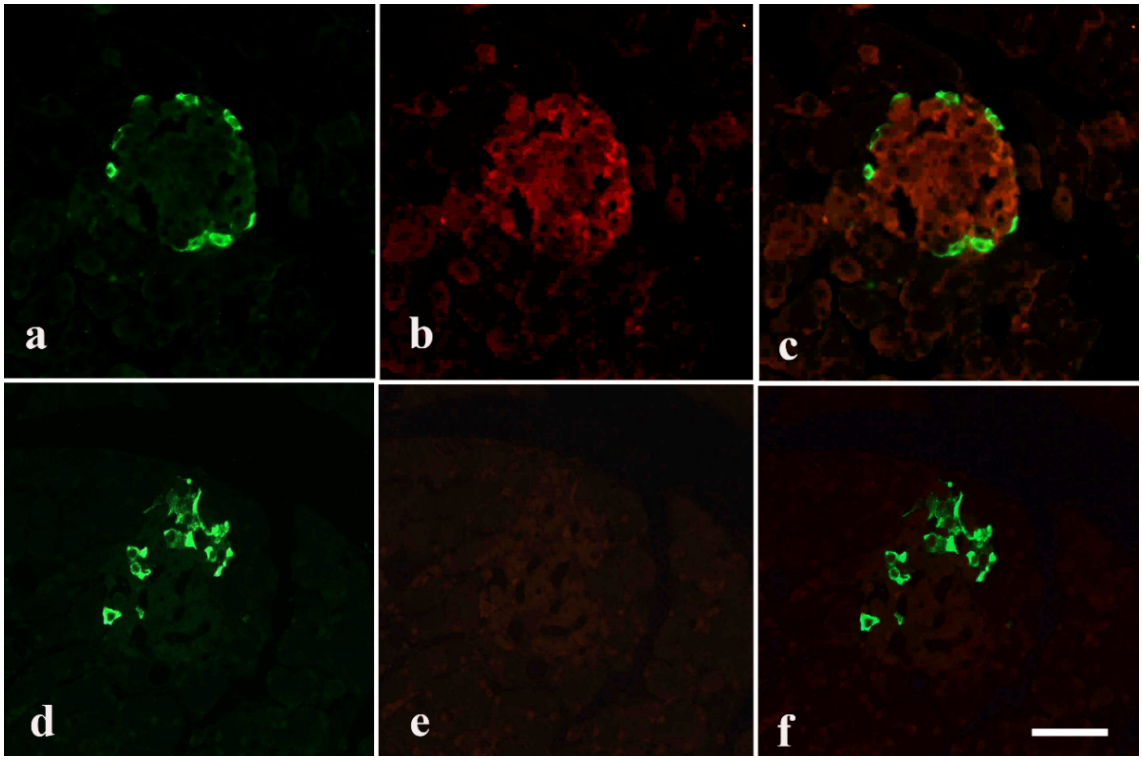


Fig. 5. Immunofluorescence images showing **(a)** somatostatin- and **(b)** NC-immunoreactive cells in the islets of control rats. Immuno-expression of somatostatin-positive cells **(d)** is significantly elevated with a concomitant reduction in the number of NC-immunoreactive cells **(e)** in islets of diabetic rats. There is no evidence of co-localization between somatostatin and NC in the islets of Langerhans. **(c** and **f** are merged IF images of somatostatin (green) and NC (red)). n = 6. Scale bar = 50 µm.
*(From Adeghate et al., Cell & Tissue Research **374**:517–529, 2018)*

6.2.4. NC and PP in the islets of Langerhans

Double labeling IF was used to determine whether NC co-localizes with PP in the islet of Langerhans. In normal rat islets, PP-positive cells were observed in the outer part of the islets, while NC-immunoreative cells, occupy the central region of the islet.

However, after the onset of DM the number and pattern of distribution of PP-immunopositive cells are altered. The number of PP-immunoreactive cells increased significantly ($p < 0.0001$) in DM rats (Fig. 6.), while PP-positive cells are now located in the central part of the islet instead of the peripheral location seen in normal rats (Fig. 6.).

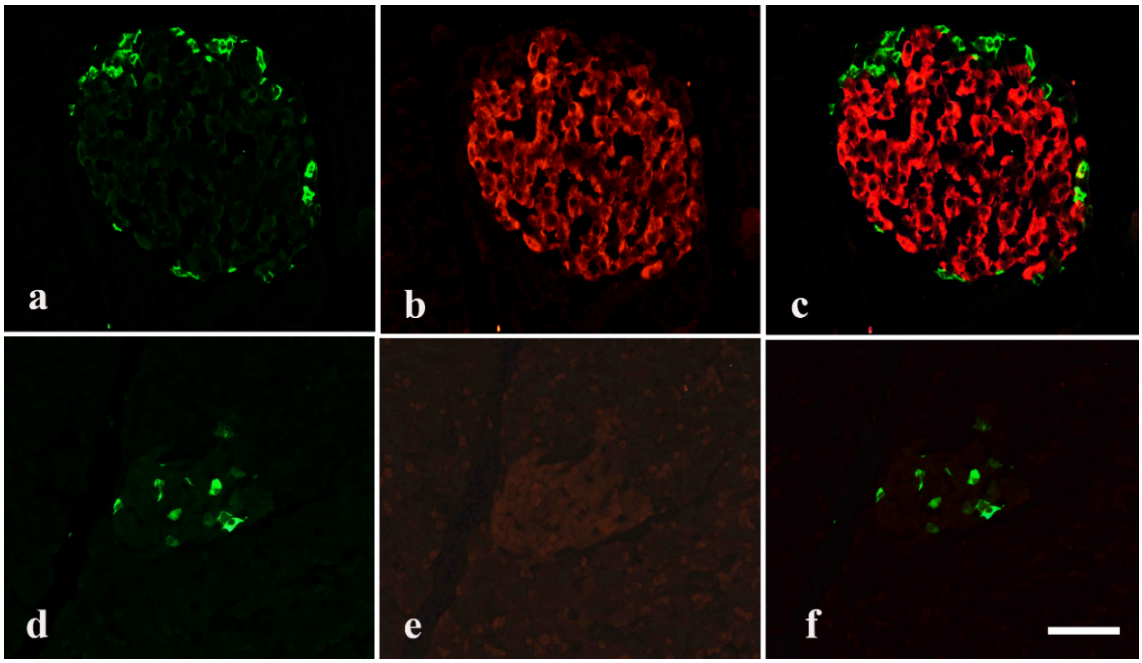


Fig. 6. Images discerning **(a)** PP- and **(b)** NC-positive cells in the islets of Langerhans control rats. Immuno-expression of PP **(d)** is elevated while that of NC **(e)** shows marked reduction in diabetic rat islets. No co-localization was observed between PP and NC **(c** and **f** are merged IF images of PP (green) and NC (red)). n = 6. Scale bar = 50 µm. *(From Adeghate et al., Cell & Tissue Research **374**:517–529, 2018)*

6.3. Immunoelectron microscopy of NC in pancreatic islet cells

Double labelled IF showed that NC co-localizes with insulin in pancreatic islet cells. IEM (Immunoelectron microscopy) was used to examine the exact location of NC in pancreatic beta cells. Immunogold particles (5 and 10 nm size) attached to IgG were used to determine the degree of co-localization of NC and insulin in insulin-producing beta cells of the pancreas. TEM of the ultrathin sections processed for immunoelectron microscopy showed NC particles (10 nm) in the secretory granules of pancreatic beta

cells with that of anti-insulin 5 nm gold particles. The presence of 5 nm-immunogold particles against insulin and that of 10 nm labelled antibodies against NC on the secretory granules of insulin-producing beta cells showed that NC and insulin are indeed co-localized at the ultrastructural level (Fig. 7.). After the onset of DM, the number of 5 nm gold particles (directed against insulin) and 10 nm gold particles (conjugated to NC) was significantly ($p < 0.02$) reduced.

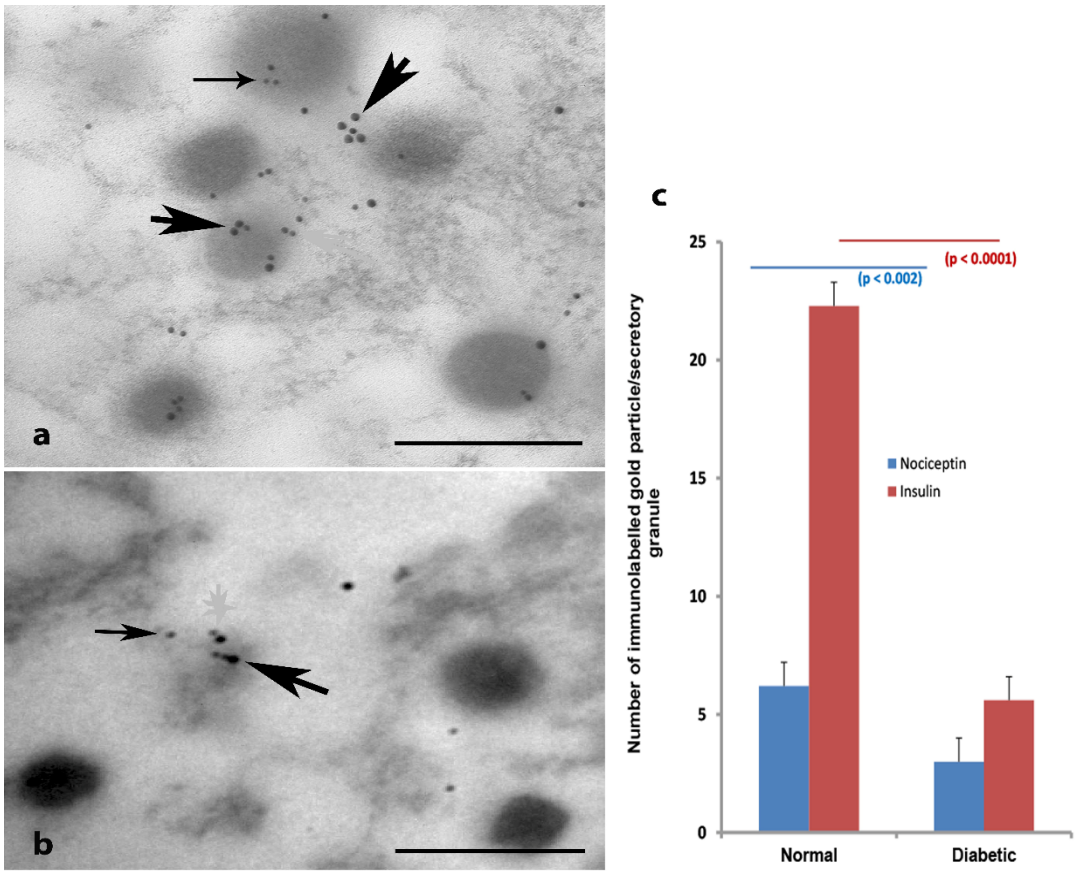
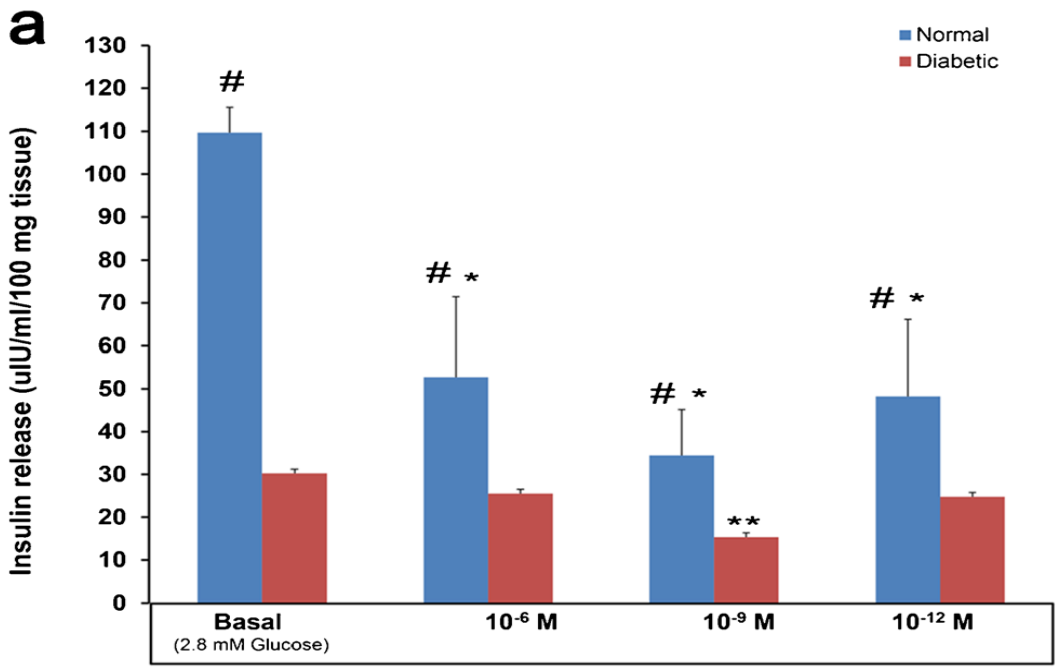


Fig. 7. Immunoelectron microscopy image of endocrine of pancreatic beta cell of non-diabetic **(a)** and diabetic **(b)** rat islets discerning insulin-labelled immunogold particles (5 nm, thin arrow) and NC (10 nm, thick arrow) on the secretory granules of pancreatic beta cells. It is worth noting that insulin and NC co-localize to secretory granules of beta cells. The number of insulin- and NC-labelled gold particles for each secretory granule was markedly reduced after the onset of DM **(c)**. n = 6. Scale bar = 1 µm. *(From Adeghate et al., Cell & Tissue Research* ***374****:517–529, 2018)*

6.4. Effect of NC on insulin and glucagon release

NC inhibited insulin release from pancreatic tissue fragments of control rat at all concentrations (10^{-12}, 10^{-9}, 10^{-6} M). NC also inhibited insulin secretion from pancreatic tissue fragments of diabetic rats. The inhibitory effect of NC was most notable ($p < 0.01$) when 10^{-9} M was used and least effective after incubation with 10^{-6} M of NC (Fig. 8a.). Incubation of isolated pancreatic islets with NC at 10^{-12}, 10^{-9}, 10^{-6} M caused a large ($p < 0.04$) reduction of insulin secretion in control rats. However, NC (10^{-12} M) induced, marked ($p < 0.02$) elevation in insulin release from islets of DM rats (Fig. 8b.). NC caused marked ($p < 0.001$) glucagon release from the pancreatic tissue fragments in normal but not in DM rats (Fig. 8c.).



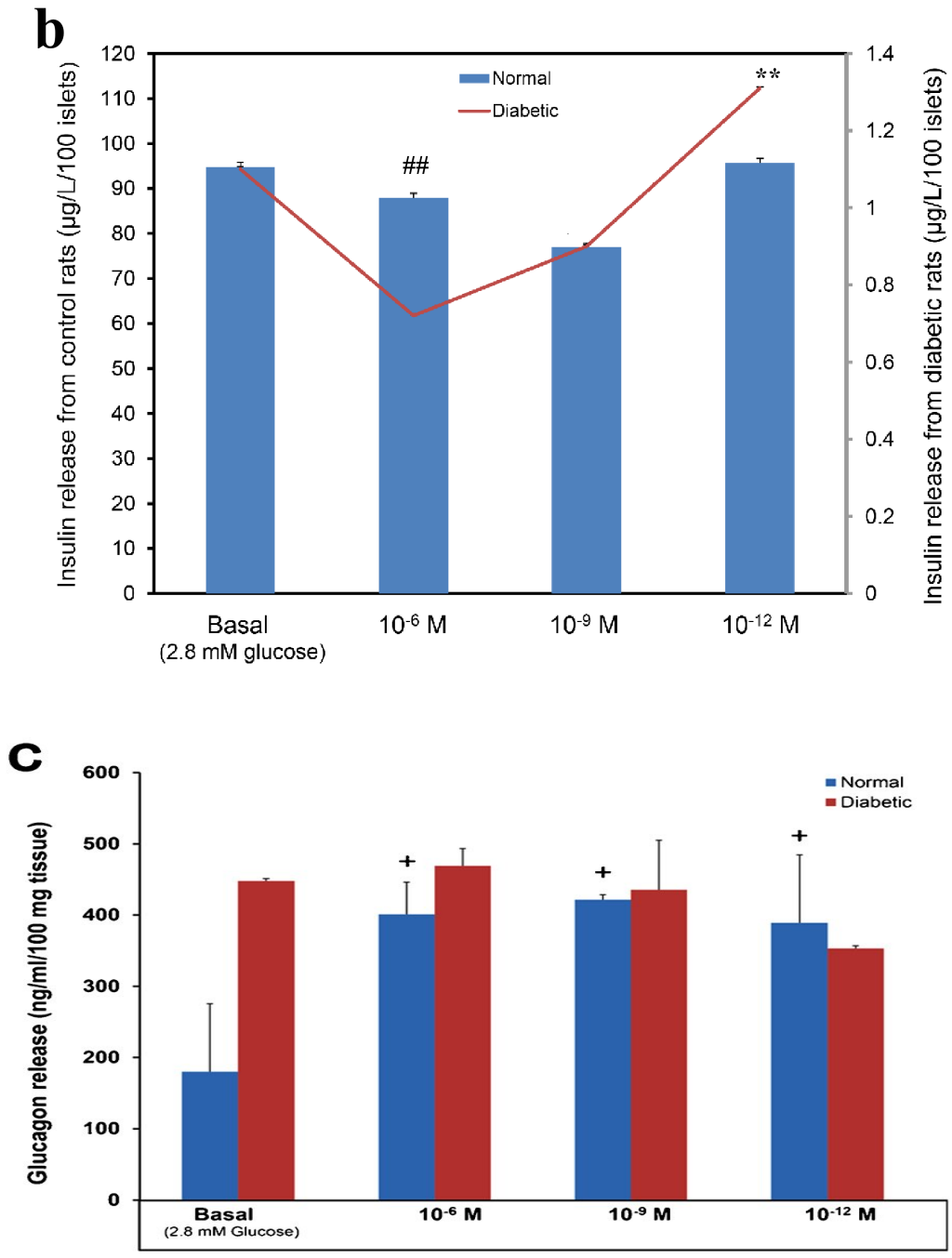


Fig. 8. The role of NC in insulin release from non-diabetic (normal) control and DM rats. NC elicited marked and significant inhibition in insulin release from pancreatic tissue fragments of normal and DM rats (*$p < 0.05$; **$p < 0.02$) (a). NC, at 10^{-6}-10^{-9} M, significantly (*$p < 0.03$) inhibits insulin release from isolated pancreatic islets of normal and diabetic rats **(Fig. 8a.)**. In contrast, NC stimulates (++$p < 0.02$) insulin release at concentration of 10^{-12} M in DM rat islets **(Fig. 8b.)**. Insulin release from pancreatic tissue fragments and islets of diabetic rats was significantly (#$p < 0.05$ fragments; ##$p <$ 0.0003 islets) reduced compared to normal controls (a, b). NC stimulates (+$p < 0.007$) glucagon from normal rat pancreas but not from diabetic rats **(Fig. 8c.)**. n = 6. *(From Adeghate et al., Cell & Tissue Research **374**:517–529, 2018).*

6.5. Effect of NC on endogenous antioxidants in renal cortex

The immune-expression of three key endogenous antioxidants, catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GRED) was examined in the renal cortex. CAT was observed in the proximal (PCT) and distal (DCT) tubules of non-diabetic and DM rats. The IF staining intensity of CAT was markedly reduced in NC-treated non-diabetic and DM rats compared to untreated control rats (Fig. 9a. - Fig. 9b.). The immune-expression of SOD increased in normal, non-diabetic rats treated with NC. In contrast, the immune-expression of SOD was significantly reduced in DM rats treated with NC (Fig. 9a. - Fig. 9c.). GRED expression in the renal cortex was markedly increased in non-diabetic, and DM treated with NC (Fig. 9a. - Fig. 9d., Fig. 10.).

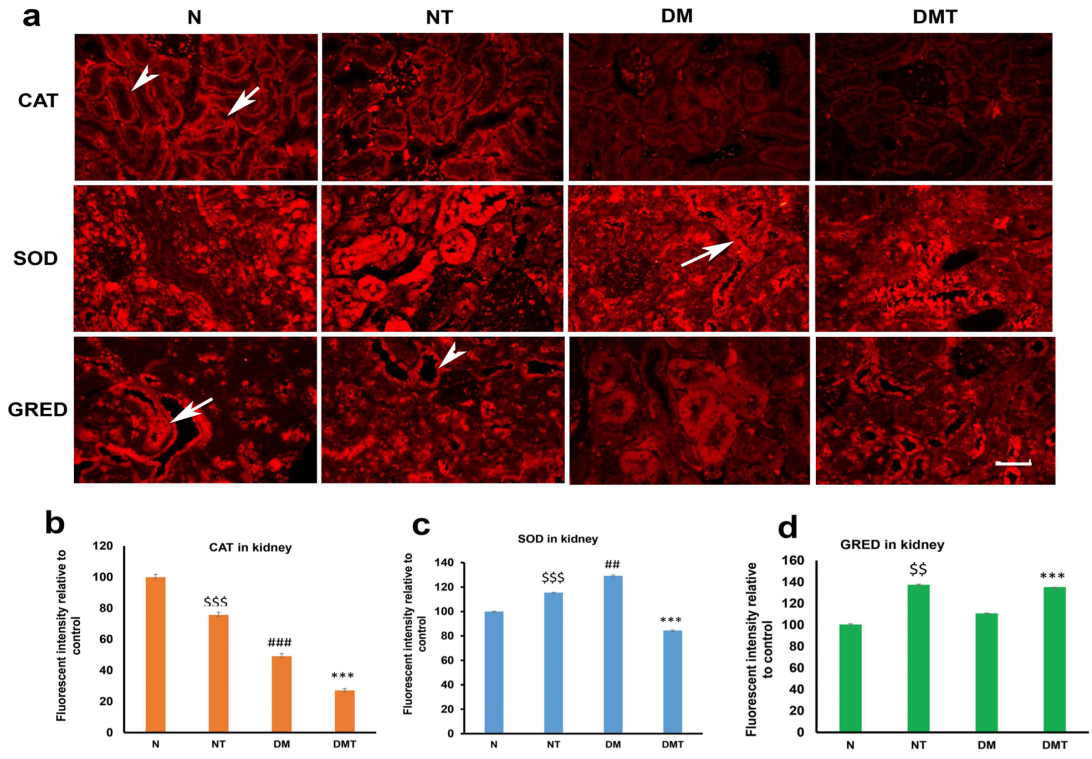


Fig. 9. **(a)** Images of IF staining of the renal cortex of the kidney of normal (N), normal NC-treated (NT), diabetic untreated (DM) and diabetic NC-treated (DMT) rats showing catalase (CAT) **(Fig. 9a. - Fig. 9b.)**, superoxide dismutase (SOD) **(Fig. 9a. - Fig. 9c.)**, and glutathione reductase (GRED) **(Fig. 9a. - Fig. 9d.)** -positive structures. Although the effect of NC treatment on the intensity of the immune-expression of CAT and SOD

is variable, the intensity of the immune-expression of GRED in normal and DM rats was significantly elevated after the administration of NC **(Fig. 9a. - Fig. 9d.)**. Proximal convoluted tubules (arrow); distal convoluted tubules (arrow head); n = 6; Scale bar = 25 µm; $$ and $$$ (normal treated versus normal untreated); # and ### (diabetic untreated versus normal untreated); *** (diabetic untreated versus diabetic treated) $$ $p < 0.05$, $$$ $p < 0.001$; *** $p < 0.001$; ### $p< 0.001$. *(From Adeghate et al., Biology **10**: 621, 2021)*

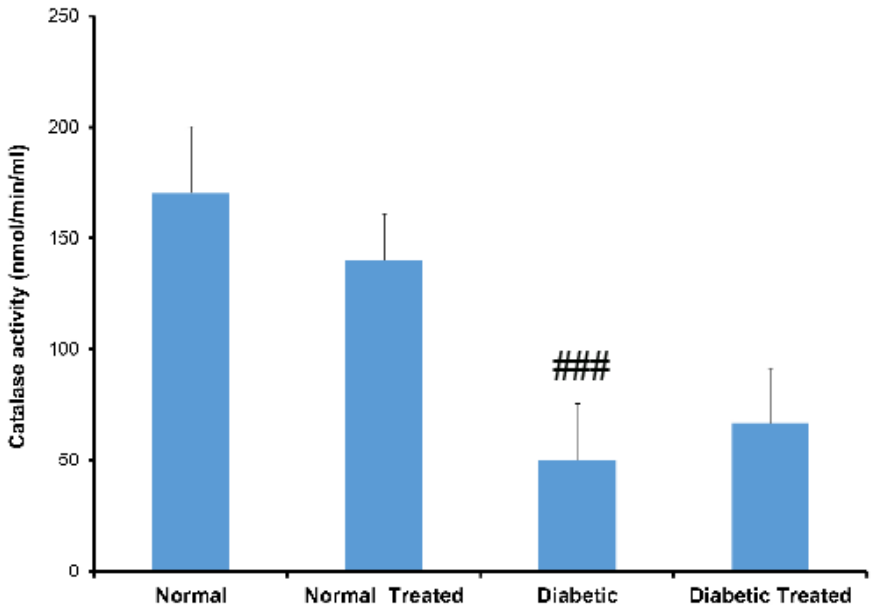


Fig. 10. Histograms of catalase (CAT) activity in the serum of normal (N), normal treated (NT), diabetic untreated (DM) and diabetic treated with NC (DMT). CAT activity was markedly reduced after the onset of DM when compared to normal. NC treatment induced a small but not significant increase in the serum activity of CAT. n = 6; ### (diabetic untreated versus normal untreated); $p < 0.01$. *(From Adeghate et al., Biology **10**: 621, 2021)*

6.6. Effect of NC on endogenous antioxidants in the liver

CAT was observed in liver cells located in the immediate vicinity of the central veins of the liver. CAT immune-expression was significantly lower in the liver of rats treated

with NC compared to controls (Fig. 11a. - Fig. 11b.). SOD-immunoreactive hepatocytes located around the central were numerous. These cells contain large quantities of SOD as evidenced from strong SOD IF staining. The tissue level of SOD immune-expression was markedly increased in both normal and diabetic rats treated with NC, when compared to untreated control rats (Fig. 11a - Fig. 11c). In addition, treated of normal and DM rats with NC resulted in the increased immune-expression of GRED in liver cells of DM rats compared to untreated controls (Fig. 11a. - Fig. 11d.).

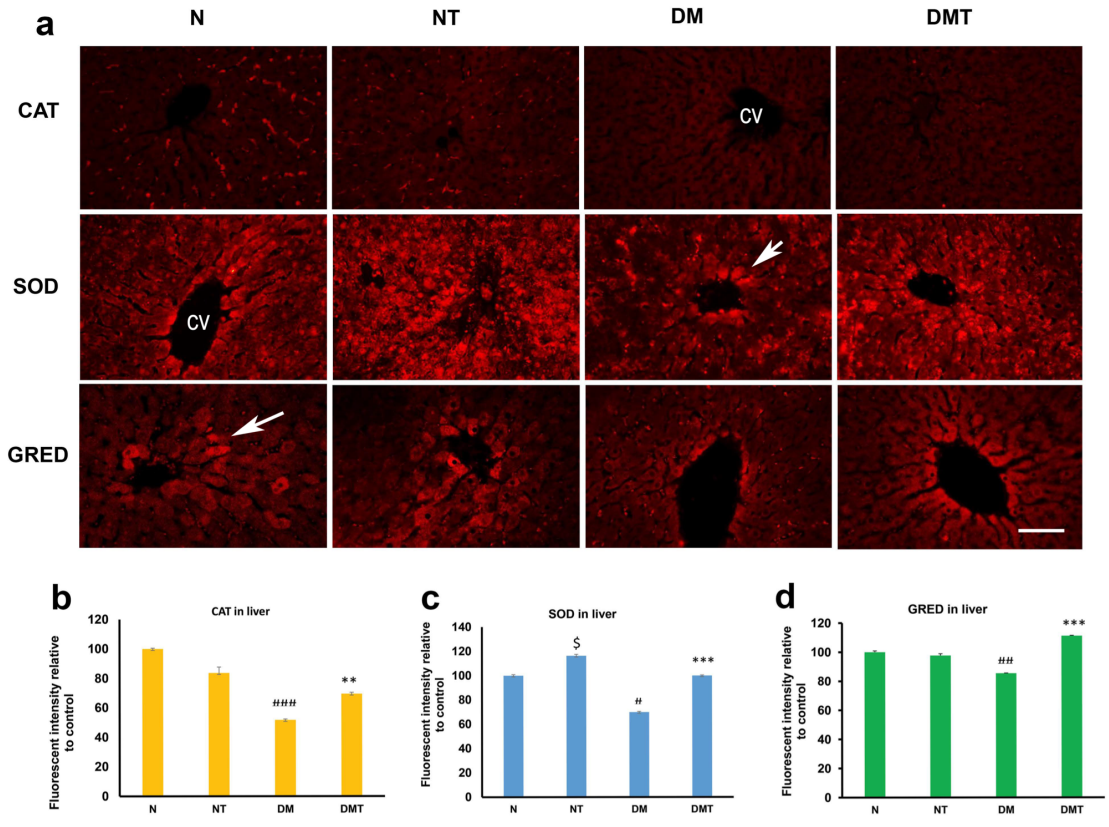


Fig. 11. IF images **(Fig. 11a.)** of catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GRED) immune-expression in hepatic cells of normal (N), normal treated (NT), DM untreated (DM), and diabetic treated with NC (DMT). The immune-expression of SOD **(Fig. 11a. - Fig. 11c.)** was significantly increased in normal and DM rats treated with NC. In addition, NC treatment caused significant increases in the hepatic level of GRED in DM rats **(Fig. 11a. - Fig. 11d.)**. cv = central vein; *n* = 6; Scale bar = 25 µm; $ (normal treated vs normal untreated); ## and ### (diabetic

untreated vs normal untreated); ** and *** (diabetic untreated vs diabetic treated). $ $p <$ 0.05; # $p <$ 0.05; ## $p <$ 0.01; ### $p <$ 0.001; ** $p <$ 0.05; *** $p <$ 0.001. *(From Adeghate et al., Biology **10**: 621, 2021)*

6.7. Effect of NC on endogenous antioxidants in the cerebral cortex

Neurons of the cerebral cortex contain CAT. The administration of NC to normal rats lowers the immune-expression of CAT, but caused a significant, increase in CAT immune-expression cerebral cortex of DM rats compared to controls (Fig. 12a. - Fig. 12b.). NC treatment did not significantly alter the immune-expression of SOD in the brain of normal rats. In contrast, treatment with NC significantly increased the SOD concentration in the neurons of the brain in DM rats. The immune-expression of SOD was markedly reduced in the brain of untreated DM rats compared to normal control rats (Fig. 12a. - Fig. 12c.). NC treatment increased GRED immune-expression in the cerebral cortex of DM rats compared to untreated DM controls (Fig. 12a. - Fig. 12d.).

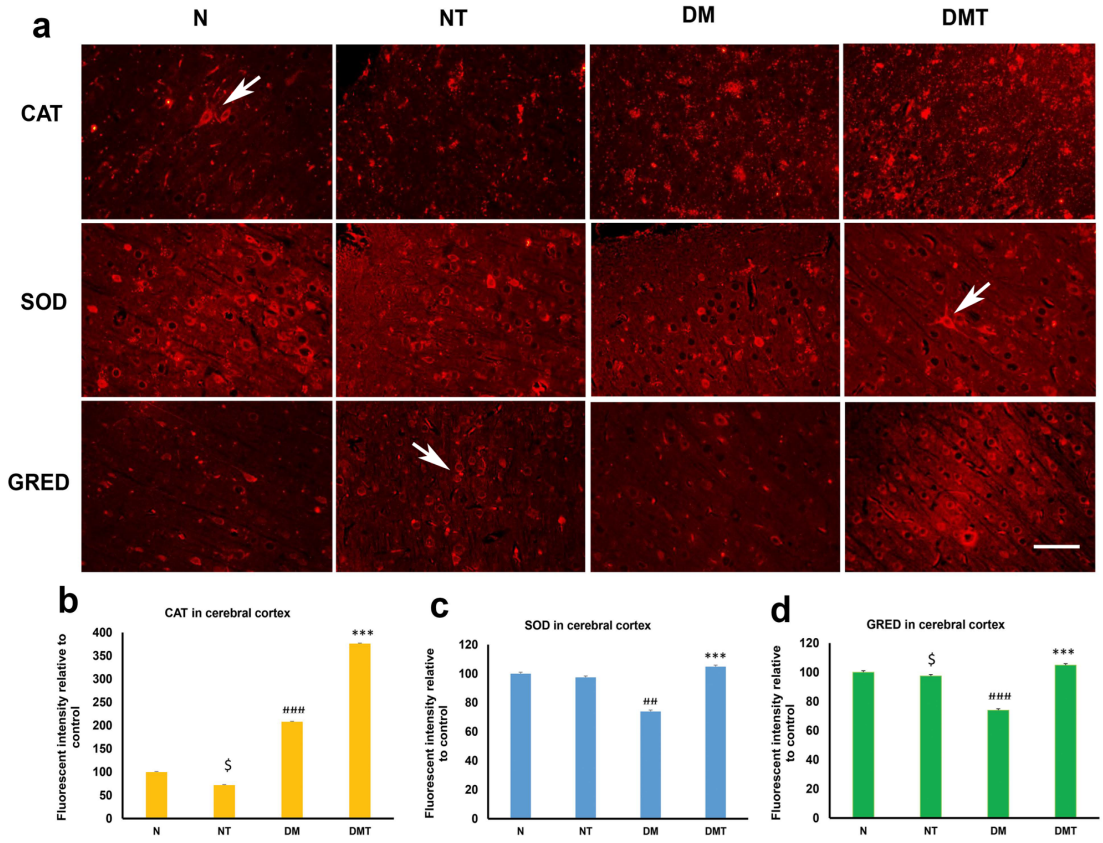


Fig. 12. IF images **(a)** of catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GRED) immune-expression of the brain of normal (N), normal treated (NT), diabetic untreated (DM) and diabetic treated with NC (DMT). It is worth noting that the immune-expressions of CAT **(Fig. 12a. - Fig. 12b.)**, SOD **(Fig. 12a. - Fig. 12c.)** and GRED **(Fig. 12a. - Fig. 12d.)** were markedly elevated in cerebral cortical neurons (arrows) of DM rats that received NC, compared to controls **(Fig . 12b., Fig. 12c. & Fig. 12d.)**. n = 6, Scale bar = 25 μm; $ (normal treated vs normal untreated); ## and ### (diabetic untreated vs normal untreated); *** (diabetic untreated vs diabetic treated) $ $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$; *** $p < 0.001$. *(From Adeghate et al., Biology **10**: 621, 2021)*

6.8. Effect of NC on endogenous antioxidants in hippocampal neurons

IF staining indicates that endogenous antioxidants can be discerned in neurons of the hippocampus. The administration of NC caused a reduction in the immune-expression of CAT in the CA3 part of the hippocampus of normal and DM rats. However, NC is unable to cause significant increases in CAT immune-expression in the CA3 region of the hippocampus of normal and DM rats (Fig. 13a. - Fig. 13b.). In contrast and when compared to controls, the immuno-expression of SOD was significantly increased in the hippocampus of normal and DM rats after treatment with NC (Fig. 13a. - Fig. 13c.). Moreover, the administration of NC induced significant increases in the immune-expression of GRED in hippocampal neurons of both normal and DM rats (Fig. 13a. - Fig. 13d.).

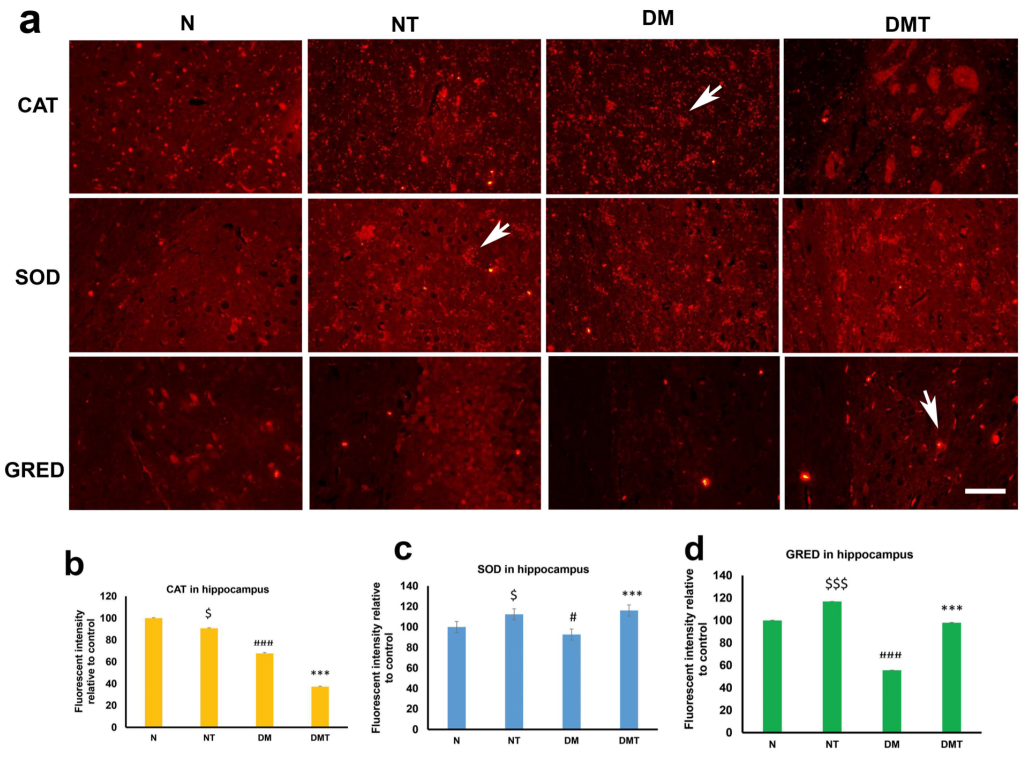


Fig. 13. IF photomicrographs **(a)** of catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GRED) immune-expression in the hippocampus (CA3 region) of normal (N), normal treated (NT), diabetic untreated (DM) and diabetic treated with NC (DMT). It is noteworthy that the immune-expressions of CAT **(Fig. 13a. - Fig. 13b.)** is reduced after NC treatment. In contrast, NC induced large and marked elevation in SOD **(Fig. 13a. - Fig. 13c.)** and GRED **(Fig. 13a. - Fig. 13d.)** immuno-expression in hippocampal neurons (arrows) of both normal and DM rats, compared to controls **(Fig . 13b., Fig. 13c. & Fig. 13d.)**. n = 6, Scale bar = 25 μm; $ and $$$ (normal treated vs normal untreated); # and ### (diabetic untreated vs normal untreated); *** (diabetic untreated vs diabetic treated) $ $p < 0.05$; $$$ $p < 0.01$; ### $p < 0.001$; *** $p < 0.001$. *(From Adeghate et al., Biology **10**: 621, 2021)*

6.9. Effect of NC on endogenous antioxidants in pancreatic islet cells

IF staining was used to determine whether the administration of NC would alter the immune-expression of catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GRED) in the islets of Langerhans.

6.9.1 Effect of NC on CAT immuno-expression pancreatic in islet cells

After NC treatment, double labelled IF of the expression of CAT was performed in conjunction with insulin.

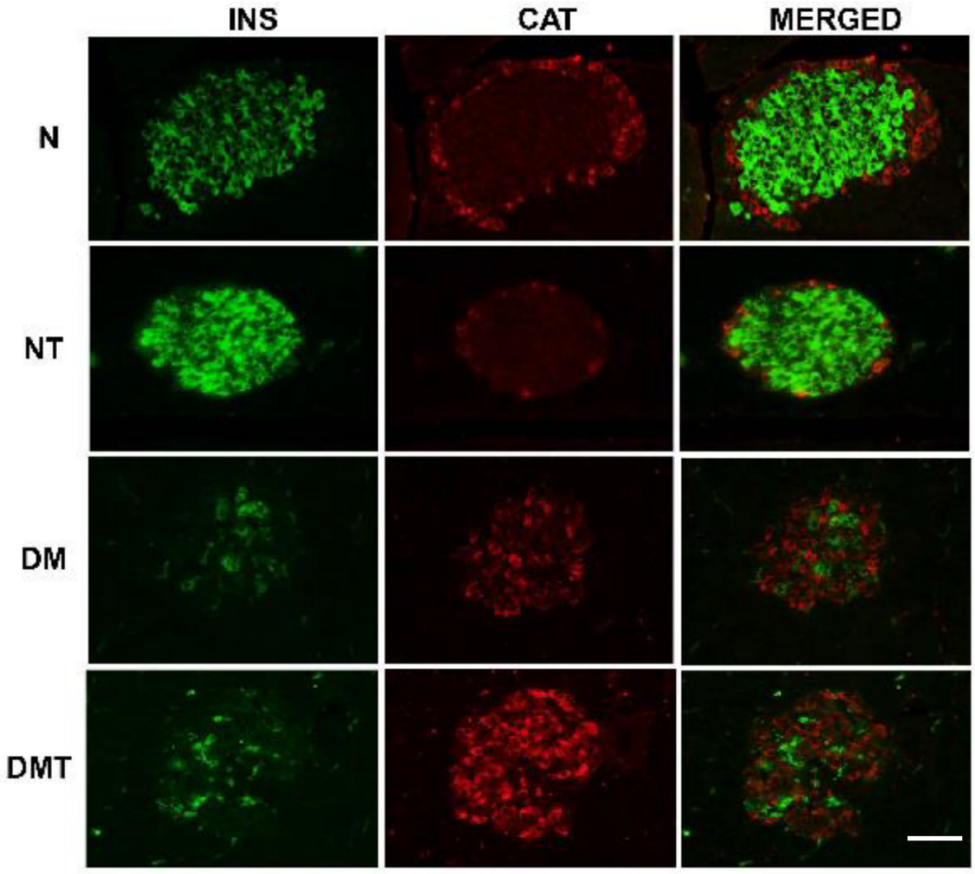


Fig. 14. IF photoimages of insulin (INS – green) and catalase (CAT - red) immunopositive cells in the islet of Langerhans. Note that the number of INS- and CAT-positive cells increased significantly ($p < 0.05$) in the islets of DM rats treated with NC. The merged images show that INS does not co-localize with CAT in pancreatic islet cells. The number of CAT-positive endocrine cells in the islet of Langerhans of DM rats increased significantly ($p < 0.05$) after NC treatment. INS does not co-localize with

CAT in the islets of either normal or diabetic rats. NC treatment did not modify the nature of co-localization of these two proteins. n = 6. Scale bar = 50 μm.
Unpublished data.

6.9.2 Effect of NC on SOD immuno-expression pancreatic in islet cells

Superoxide dismutase (SOD) was observed in the central region of pancreatic islets of normal rats. These SOD-containing cells co-localizes with insulin (INS) (Fig. 15). The number of INS and SOD-immunoreactive cells decreased significantly after the onset of DM. However, the percentage number of SOD increase substantially after treatment with NC.

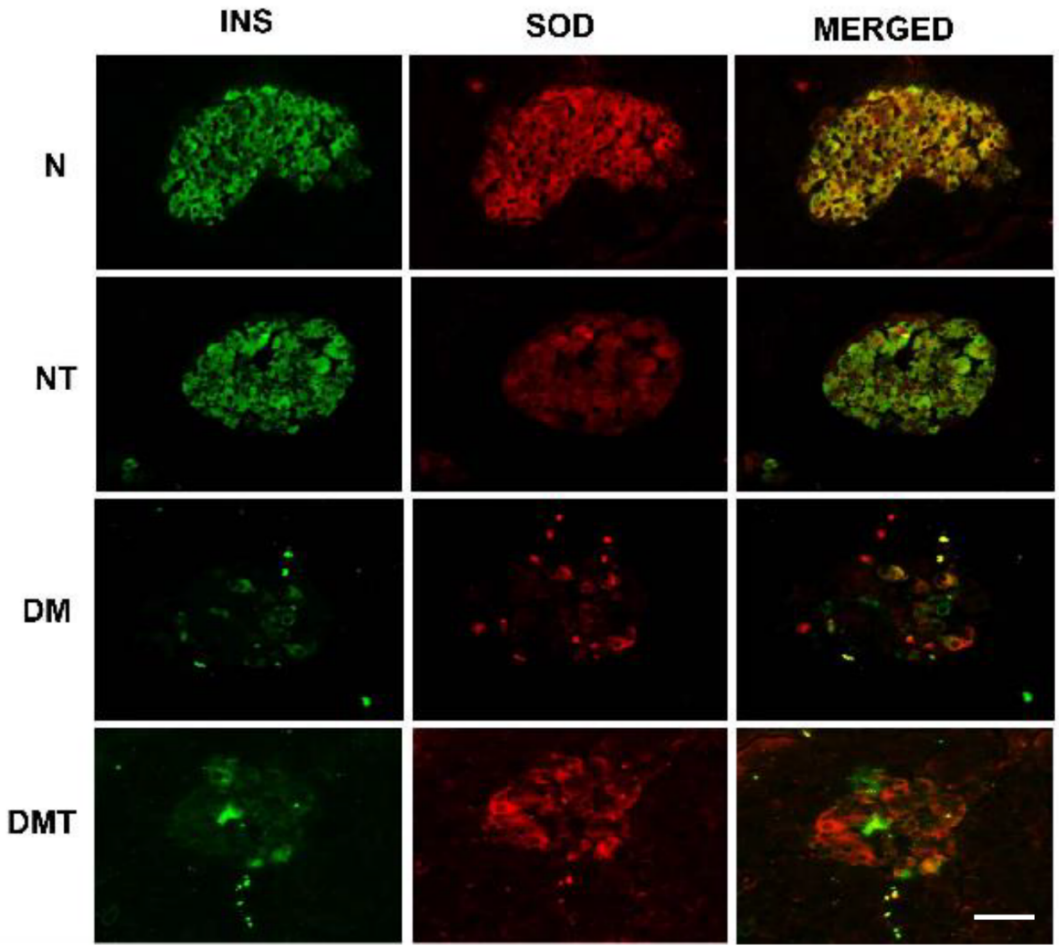


Fig. 15. IF photoimages of insulin (INS-green) and superoxide dismutase (SOD - red) immunoreactive cells in pancreatic islets. Note that the number of INS- and SOD-immunopositive cells increased significantly ($p < 0.05$) in the islets of DM rats treated

with NC. A review of the merged images shows that SOD co-localizes with INS in the islets of Langerhans. n = 6. Scale bar = 50 μm. *Unpublished data.*

6.9.3 Effect of NC on GRED immuno-expression pancreatic in islet cells

Glutathione reductase (GRED), another key antioxidant is located in cells located in the central part of the islet of Langerhans. In pancreatic islets of normal and DM rats, INS and GRED co-localize. NC treatment significantly increased the number of INS and GRED-immunoreactive cells in the endocrine pancreas (Fig. 16.).

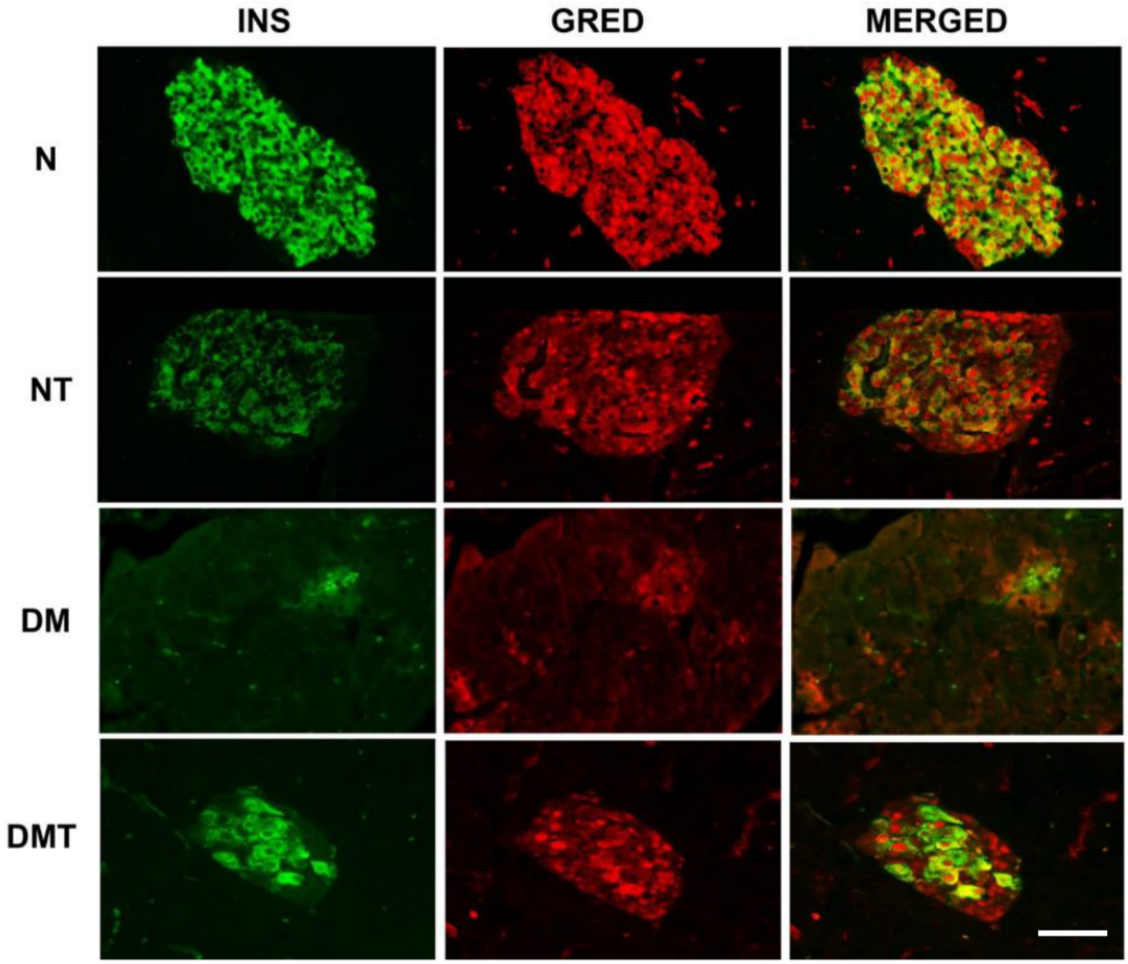


Fig. 16. IF photoimages of insulin (INS - green) and glutathione reductase (GRED - red) immunopositive cells in pancreatic islets of Langerhans. Note that the number of INS- and GRED-immunoreactive cells increased markedly ($p < 0.05$) in the islets of DM rats treated with NC. A review of the merged images shows that GRED co-localizes with INS in the islets of Langerhans. n = 6. Scale bar = 50 μm. *Unpublished data.*

7. Discussion

7.1. General features of rats with experimental diabetes mellitus

7.*1.1. Body weight*

During the period of study that spans over 4 weeks, diabetic Wistar rats showed significant loss of body weight compared to non-diabetic normal control rats. The loss of weight is probably due to the destruction of pancreatic beta cells leading to low plasma insulin level. Since insulin is required for the uptake of glucose into hepatic, fat and skeletal muscle cells, the amount of glucose uptake into these cells will be very low in diabetic rats. The low glucose content in these cells would not be enough to drive the metabolism that is required by these energy-consuming cells. These cells especially the active skeletal muscle cells will burn protein instead of glucose, leading to muscle waste and body weight loss. The phenomenon of weight reduction in STZ-induced diabetes has been reported in the literature [68].

7.*1.2. Blood glucose*

The blood glucose level of diabetic rats increased significantly right from the onset of diabetes to the end of the experiment. This hyperglycemia is a hallmark of diabetes mellitus. The reason behind diabetes-induced hyperglycemia is the destruction of insulin-producing beta cells in the endocrine pancreas. The loss of insulin leads to impaired glucose uptake from blood circulation to peripheral cells such as skeletal muscle, hepatic as well as liver cells from the blood, leaving excess glucose in the blood. The mechanisms by which STZ destroys the beta cells of the pancreas has been previously described. STZ binds to GLUT2 transporter before entering beta cells. This signaling process is then followed by the destruction of pancreatic beta cell [69]. Destruction of pancreatic beta cells is facilitated STZ-induced increases in cellular poly-ADP-ribose polymerase enzyme (PARP), which exhaust nicotinamide adenine dinucleotide (NAD) cellular reserve [70]. Therefore, the increased blood glucose level

is caused by low insulin level due to damage of pancreatic beta cells, caused by the depletion of NAD.

7.2. NC in the cells of the islet of Langerhans

7.2.1. NC and insulin-positive cells

In this study we used double-labeling immunofluorescence to study whether NC is found in the same cells with either insulin, glucagon, somatostatin, or PP. Our investigation showed that NC-immunopositive cells can be seen in the inner core of pancreatic beta cells, where insulin-producing cells are normally located. Double labelling immunofluorescence study confirmed that NC co-localizes with insulin in the beta cells of the endocrine pancreas. It was noteworthy to observe that the number of NC-immunoreactive cells was significantly reduced after the onset of DM. This is a feature that is akin to beta cell. The fact that NC is found in the central region of the islet of Langerhans, diminished in number after the induction of DM, and the merging together of NC and insulin, shows that NC and insulin are co-localized and intimately located in pancreatic beta cells. Image analysis confirm that the number of NC-positive islet cells was significantly reduced in diabetic rats compared to controls. This observation corroborate the findings of Tariq et al. [71]. The large and marked depletion in the number of NC-immunoreactive islet cells with a concurrent reduction in the number of insulin-containing pancreatic islet cells indicates that DM is associated with a reduced number of NC-containing cells in the endocrine pancreas. It is not known whether, this reduction in the NC content of in the endocrine pancreas contributes to the neuropathic pain observed in diabetic patients. Previous studies have shown that activation of the nociceptinergic system actually inhibits hyperalgesia in experimental diabetic neuropathy [72].

7.2.2. NC and glucagon-positive cells

We showed that NC co-localized with insulin in the most of the pancreatic beta cells of the endocrine pancreas. Our other objective was to determine whether, this co-localization is also true for glucagon. Using, double-labeling IF technique, no co-localization was observed between NC and glucagon in pancreatic islet cells of Langerhans of either normal or diabetic Wistar rats. Most of the NC-positive endocrine cells were discerned in the core of the islets while glucagon containing endocrine cells were located in the periphery of the islets. In addition, the number of NC-positive cells was significantly higher than that of glucagon-immunoreactive cells. Moreover, the number of NC-positive cells decreased after the onset of DM while that of glucagon increased. All of these observations indicates that NC do not physically reside with glucagon in a single cell. The increase in the number of glucagon positive cells serves another purpose rather, which is the enhancement of the level of plasma glucagon as seen in uncontrolled DM [73].

7.2.3. NC and somatostatin-positive cells

We also wanted to know whether NC co-localized with somatostatin, another major pancreatic hormone. Double-labelling IF showed that somatostatin-positive cells are located in the peripheral region of the pancreatic islets whole NC-positive cells were seen in the central portion of the endocrine pancreas of normal rat. However, this pattern of distribution is disrupted after the onset of DM, with a large reduction in the number of NC-containing cells and increase in the number of somatostatin-positive cells. In addition, the pattern of distribution of NC and somatostatin changes after the induction of DM, where it not only occupies the peripheral parts of the islets but also the core. Similarly to glucagon, this findings indicate that NC and somatostatin are not likely to reside in one and the same endocrine cell. We showed that NC does not co-localize with somatostatin in the islets of Langerhans, indicating that NC may directly regulate the metabolism of somatostatin. The significant increase in the number of somatostatin-containing cells observed after the induction of DM has been reported previously [74].

7.2.4. NC and PP-positive cells

In a similar trend with glucagon and somatostatin, double labeling IF showed that the location of NC- and PP-positive cells are different within the endocrine cells of the islet of Langerhans. NC are located in the core of the islets in both normal and DM rat pancreas, while PP-positive cells are discerned mainly in the periphery of the islets of normal, non-diabetic rats and in both the core and outer parts after the induction of DM. In addition to changes in the pattern of PP-immunopositive cells seen after the onset of DM, the number of PP-immunoreactive cells increased significantly in the islet of Langerhans of DM rats. Our study clearly showed that NC does not co-localize with PP in the pancreatic endocrine cells of either normal or diabetic rats. This suggests that NC may not directly influence PP metabolism by „contact" within the same islet cell.

7.3. Transmission electron microscopy of NC in the endocrine pancreas

Since we have shown by IF that NC is present in pancreatic beta cells of both normal, non-diabetic rats, we were curious to determine the exact location of NC in these cells. Using immunoelectron microscopy we showed that NC is located on the secretory granules of beta cells of pancreatic islets. This observation regarding the presence of NC in secretory granules of pancreatic beta cells of the pancreas concurs with those of Tariq et al. [70]. This observation suggests that NC and insulin are both processed by pancreatic beta cells and could possibly be released simultaneously from secretory granules into blood circulation. It is worth noting that NC is present in the plasma. The plasma level of NC may be increased in some condition such as coronary artery disease [74]. However, it is not known how much of the plasma NC would be derived from the endocrine pancreas. In spite of this, this close relation between NC and insulin both structurally and ultrastructurally suggests that NC may likely be implicated in the regulation of insulin metabolism.

7.4. The role of NC on insulin and glucagon release

7.4.1 NC and insulin release

We showed that NC (10^{-6} -10^{-12} M) induced significant reduction in insulin release from pancreatic tissue fragment of DM rats. This reduction was most potent at 10^{-9} M of NC. It has been shown that some peptides, including galanin can inhibit insulin release rather than stimulating insulin secretion [76]. To the best of our knowledge, this is the first reported study on the effect of NC on insulin secretion. However, there are reports on the effect of opioid receptors insulin release. Tudurí et al. [77] reported that stimulation of opioid receptors inhibits insulin release. Previous literature reports have shown that opioid receptors are present in the plasma membrane of pancreatic beta cells [77, 78]. It is also possible to draw parallel conclusion with NC, since NC is capable on acting on opioid members of the receptor family [79]. NOP receptors are approximately 60% similar to classical opioid receptors [80]. It was interesting to note that, when isolated islets from diabetic rat were used, NC at a concentration of 10^{-12} M evoked a slight but significant release of insulin. The differences between these results may be due to the type of substrate used during the stimulation experiment. On one hand, whole tissue fragments were used and on the other isolated islets were stimulated.

7.4.2 NC and glucagon release

In contrast to inhibiting insulin release from pancreatic tissue fragments of non-diabetic rats, NC at different concentrations (10^{-6} -10^{-12} M) stimulated glucagon release from the pancreas of non-diabetic rats. NC did not significantly affect glucagon release from pancreatic tissue fragments of DM rats. The differences in the manner in which NC affects the release of insulin and glucagon may point to a compensatory mechanism within the endocrine pancreas for the maintenance of metabolic equilibrium. In normal non-diabetic rats the compensatory mechanism is intact; however, this process is eliminated after the onset of diabetes. Thus explaining the variation in the manner in which NC affects glucagon release.

7.4.3. NC and signal transduction in beta cell of the islets of Langerhans

We presumed that NC binds to membrane-bound NC opioid peptide receptor on pancreatic beta cells, which may inhibit ATP K^+ channel cascading into a reduced ATP [77]. Reduced ATP levels lead to a reduction in intracellular Ca^{2+} accessible for Ca^{2+}-induced granule release from beta cells. As for the beta cells of the DM rats, it is possible that the structure of membrane-bound molecules such as ATP K^+ channel protein may have been altered, leading to hypersensitivity to low concentration of intracellular glucose. In this case, NC would be able to stimulate insulin release as observed in isolated rat islets.

7.5. Effect of NC on endogenous antioxidants in the renal cortex

7.5.1. NC and CAT in kidney

Catalase (CAT) is found in many cells and protects the body by converting H_2O_2 into harmless O_2 and H_2O [81]. Our study showed that CAT is discerned in the renal cortex within the epithelium lining the proximal and distal, convoluted tubules in normal, non-diabetic rats. Our findings are in agreement with those of Johkura et al. [82]. Johkura and co-workers showed, using immunohistochemistry, that developing kidneys contains CAT. Most others studies determined the level of NC in the plasma rather than the kidney. We showed that the proximal (PCT) and distal (DCT) convoluted tubules of the kidney of normal and diabetic rats contain CAT. However, the degree of immune-expression of CAT was reduced in untreated diabetic kidney compared to untreated controls. Moreover, the intensity of the immune-expression of CAT was markedly reduced in NC-treated normal rat kidney cortex when compared to untreated normal controls. More studies are needed to be able to decipher the significance of these findings.

7.5.2. NC and SOD in kidney

Superoxide dismutase (SOD) neutralizes ROS in cells [83]. Out of the three isoforms of SOD, the cytoplasmic (SOD1) and the mitochondria (SOD2) are the most important regarding oxidative stress [84]. SOD is involved in the conversion of superoxide ions into O_2 and H_2O_2. The H_2O_2 is then "handed" over to catalase for destruction [85]. In this study, we used a "whole" SOD that contains all of the three isoforms. Treatment with NC caused large and significant elevation of the immune-expression of SOD in the PCT and DCT of renal cortex. However, the immune-expression of SOD was markedly reduced in the renal cortex of DM rats compared to those treated with NC. The reason why the cortical content of SOD is reduced is not clear. The renal content SOD may have been depleted during the course of DM. It is however, well known that chronic DM leads to kidney failure, where most of the renal structures including the PCT and DCT have structural lesions [86].

7.5.3. NC and GRED in kidney

Glutathione reductase (GRED) converts GSSG to GSH, which in turn reduces H_2O_2 to water [87]. NC when given i.p. markedly increased the immune-expression of GRED in the renal cortices of normal and DM rats. This observation suggests that NC can indeed increase the tissue level of GRED both normal, non-diabetic and DM rats. It is difficult to compare our result to that of the literature since, to the best of our knowledge, this is the first reported NC-induced increase immune-expression of GRED in the renal cortex of normal and DM rats.

7.6. NC and endogenous antioxidants in the liver

7.6.1. NC and CAT in liver

CAT immune-expression was markedly reduced in the liver after the onset of DM. The plasma level of CAT was also lower in DM rats compared to control. Our results corroborates those reported in the literature, which indicates that DM causes reduction in the plasma level of CAT compared to control [88]. The administration of NC

increased CAT immune-expression in the liver of both normal and DM rats. The authors do not have knowledge of other studies reported on the effect of NC on CAT immune-expression in hepatocytes.

7.6.2. NC and SOD in liver

The results demonstrated that immune-expression of SOD is significantly increased in liver cells of normal, non-diabetic and DM rats after NC treatment. The SOD-containing hepatocytes were observed around the central veins of the liver. We do not know why SOD-positive hepatocytes congregate around the central veins of the liver. It is probable that blood around these parts of the liver contain more free radicals because they actually receive more blood circulation compared to areas distant to them.

7.6.3. NC and GRED in liver

The results demonstrated the presence of glutathione reductase (GRED) in hepatocytes residing around the central veins of the liver. Treatment with NC significantly increased the immune-expression of GRED in hepatocytes located in the immediate vicinity of hepatic central veins. This observation was most visible in DM rats. All of these findings indicate that endogenous antioxidants, such as SOD, CAT and GRED are abundant in parenchymal cells residing around hepatic central veins. This observation suggests that endogenous antioxidants are required in this part of the liver.

7.7. NC and endogenous antioxidants in the cerebral cortex

The cerebrum houses billions of NOS-containing neurons, capable of producing NO. The cerebral cortex is protected by CAT, SOD and GRED. Previous reports showed that the brain contains large quantities of CAT, SOD and GRED. The tissue levels of these enzymes are said to be very high in developing brain [89, 90], where they were reported to be able to work together to destroy H_2O_2 in brain culture cells [91].

7.7.1. NC and CAT in cerebral cortex

Treatment of normal and DM rats with NC caused significant increase in immuno-expression of CAT in cortical neurons of the brain when compared to untreated controls. The NC-induced increase in the immune-expression of CAT in the neurons of the cerebral cortex is most pronounced in DM rats. The reason for these findings is not clear, however, the brain level of CAT in DM rats when compared with of normal non-diabetic controls, leading to an urge for the brain to compensate for this abnormally low tissue level of CAT. This phenomenon may be an upregulation in NC-treated DM rats against a negative feedback in NC-treated normal rats. Our results corroborate that of a recent report in which a herbal medicine was used to stimulate the level of CAT in the cerebral cortex of DM rats [92].

7.7.2. NC and SOD in cerebral cortex

The presence of SOD in cerebral neurons is a logical response to the prominent occurrence of oxidative stress in the neurons of the cerebral cortex. SOD is needed to neutralize toxic effects of reactive oxygen species generated during cellular metabolism in neurons. DM caused a significant reduction in the immune-expression of SOD compared to non-diabetic control. However, NC treatment caused large and significant increases in the immune-expression of SOD in the cerebral neurons of DM rats, compared to untreated DM control. This indicates that NC can indeed stimulate increases in tissue level of SOD.

7.7.3. NC and GRED in cerebral cortex

DM caused significant depletion of GRED in cerebral neurons compared to non-diabetic controls. NC treatment induced a reduction in the immune-expression of GRED in cerebral neurons of non-diabetic rats. In contrast, NC treatment caused significant increases in the immune-expression of GRED in cerebral cortical neurons of DM rats compared to untreated diabetic controls. A similar investigation demonstrated that melatonin increased the tissue concentration of GRED in the brain of DM rats [93]. These findings suggest that NC is a potent inducer of endogenous antioxidants.

7.8. NC and endogenous antioxidants in the CA3 region of the hippocampus

The *cornu ammonis* 3 or simply the CA3 area of the hippocampus is situated in the dorsal part of the hippocampus. This area of the hippocampus plays a crucial role in the maintenance of memory [94]. We investigated whether NC treatment can alter the immuno-expression of CAT, SOD and GRED in this important territory of the hippocampus. Moreover, NC has been implicated in physiological functions, including learning and memory that occur primarily in the hippocampus [51]. Since oxidative stress has been implicated as a cause of many neurological diseases [95], the need to increase the tissue level of intrinsic antioxidants such as CAT, SOD and GRED cannot be over emphasized.

7.8.1. NC and CAT in the hippocampus

The immune-expression of CAT was significantly reduced after the onset of DM. This finding is in agreement with other studies on different parts of the brain [51, 95]. Unexpectedly, the immune-expression of CAT was markedly lower after i.p. injection of NC to either normal or DM rats. The reason for this observation is unknown. It may be due to technical or other issues.

7.8.2. NC and SOD in the hippocampus

The administration of NC caused large and significant increase in the immune-expression of SOD in non-diabetic and DM rats. This may indicate that the tissue level of SOD can be enhanced by NC in the hippocampus.

7.8.3. NC and GRED in the hippocampus

In a similar trend with that of SOD, GRED immune-expression in hippocampal neurons increased significantly after NC treatment. This indicates that GRED is present in this crucial region of the brain and its level can be enhanced with exogenous peptide such as NC.

7.9. NC and endogenous antioxidants in the endocrine pancreas

7.9.1. Effect of NC on insulin and CAT in islet cells

Insulin is localized in the central portion of pancreatic islets while CAT is discerned in the cells sitting at the periphery of the islets. This pattern of distribution suggests that CAT would co-localize with glucagon while NC co-localizes with insulin. We have shown at the light and electron microscopy level that NC is found in insulin-producing beta cells of the pancreas. The effect of NC treatment on the immuno-expression CAT is not clear cut. NC treatment reduced the number of CAT-positive cells in the islets of normal pancreas. In contrast, treatment of DM rats with NC significantly increased the number of insulin- and CAT-immunoreactive cells in the islets of Langerhans. Again, to the best of our knowledge this is the first reported study on the effect of NC on the number of CAT-positive cells in the endocrine pancreas.

7.9.2. Effect of NC on INS and SOD in islet cells

Insulin- and SOD-positive cells are localized to the central portion of pancreatic islets of normal non-diabetic rats. The number of insulin- and SOD-containing cells diminished significantly after the induction of DM. Although NC treatment did not induce significant increase in the number of SOD-positive cells in the islet of normal rats, however, it caused large and marked increases in the number of INS- and SOD-containing cells in DM rats.

7.9.3. Effect of NC and GRED in islet cells

Insulin- and GRED-imunoreactive cells were observed in the core the islets of normal rats. In a similar manner to that of SOD, the number of INS- and GRED-immunopositive cell was significantly reduced after the onset of DM. NC treatment caused a large and marked increases in the number of INS- and GRED-immuno-positive cells in pancreatic islets of DM rats. All these shows the ability of NC to increase the number of islet cells that contains these important endogenous antioxidants.

8. Conclusion

We concluded that NC is present in the cells occupying a significant part of the core of the islets of Langerhans of non-diabetic rats. The number of these NC-immunoreactive cells is significantly reduced after the onset of DM. Double labelling immunofluorescence showed that NC co-localizes with insulin in the endocrine cells of normal and diabetic rats. NC does not co-localize with either glucagon, somatostatin, or PP cells in pancreatic islets. Using transmission electron microscopy, we showed that NC is present in the secretory granules of insulin-secreting beta cells of the pancreas of normal and DM rats. NC was able to markedly inhibit insulin secretion from pancreatic tissue fragments of normal and DM rats. In contrast, NC stimulates insulin secretion from isolated islets of DM rats. All of these observations show that NC is indeed present in insulin-producing pancreatic beta cell and takes part in insulin secretion.

Moreover, we showed that intraperitoneal treatment of normal and diabetic rats with NC significantly increased the immune-expression of SOD and GRED in the pancreas, kidney, liver, and neurons of the cerebral cortex and hippocampus. This NC-induced induction of endogenous antioxidants was especially prominent in DM rats. Therefore, NC may be exerting its physiological and neuroprotective effects by enhancing the expression of endogenous antioxidants in different cell types including neurons.

9. Summary

Background: Nociceptin (NC), which was discovered in 1995, is a small (17-amino acids) endogenous peptide. It was first observed in the central nervous system, but also located in other organ systems. The NC ligand acts on nociceptin opioid peptide receptor to modulate a large variety of physiological functions including but not limited to nociception, neurotransmitter and hormone release, and differentiation of neurons and many others. **Aims and objectives:** Our aim was to investigate the cellular localization of NC in the pancreas and to determine whether NC can stimulate hormone release from the islet of Langerhans of both normal and diabetic rats. We also wanted to know if NC could increase the immuno-expression of endogenous antioxidants in normal and diabetic rats. **Methods:** Male Wistar rats weighing 250 g were used in this study. Diabetes mellitus (DM) was induced with streptozotocin 60 mg/kg body weight. Double labelling immunofluorescence (DIF) and transmission immune-electron microscopy (TIEM) techniques were used to determine the cellular and ultrastructural localization of NC in islet cells, and to determine whether NC co-localizes with pancreatic hormones or induce the immune-expression of endogenous antioxidants. Non-diabetic and DM rats were treated with NC to determine whether NC can stimulate hormone secretion from isolated pancreatic tissues. **Results and Discussion:** Our study showed that NC is present in pancreatic islet cells. The number of NC-immunoreactive cells was significantly ($p < 0.05$) reduced after the onset of DM. DIF technique showed that NC co-localizes with insulin, but not with either glucagon, somatostatin, or pancreatic polypeptide. TIEM showed that NC is present in the secretory granules of insulin-secreting beta cells of the pancreas of normal and DM rats. All of these observations show that NC is present in insulin-producing pancreatic beta cell and takes part in insulin secretion. Intraperitoneal injection with NC significantly increased the immuno-expression of SOD and GRED in the pancreas, kidney, liver, and neurons of the cerebral cortex and hippocampus. **Conclusion:** NC may be exerting its physiological and protective effects by enhancing the expression of endogenous antioxidants in different cell types including pancreatic beta cell.

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11. Bibliography of the candidate's publications

11.1. Publications related to the PhD thesis

11.1.1. Full Length Articles

[1] **Adeghate E**, Saeed Z, D'Souza C, Tariq S, Kalász H, Tekes K, Adeghate EA. Effect of nociceptin on insulin release in normal and diabetic rat pancreas. Cell Tissue Res. 2018; 374: 517-529 [Impact factor: 3.360]

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11.1.2. Abstracts

[1] **Adeghate E**, Tekes K. Distribution of nociceptin-immunoreactive nerves in the dorsal root ganglion of GK rats. The FASEB J 2016; 30(Suppl. 1): lb35- lb35

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11.2. Publications not related to the PhD thesis

[1] Kalász H, Ojha S, Tekes K, Szőke E, Mohanraj R, Fahim M, **Adeghate E** and Adem A. Pharmacognostical sources of popular medicine to treat Alzheimer's disease. The Open Med Chem J 12: 23-35, 2018 [Impact factor: 0]

[2] **Adeghate E**, Mohsin S, Adi F, Ahmed F, Yahya A, Kalász H, Tekes K, Adeghate EA. An update of SGLT1 and SGLT2 inhibitors in early phase diabetes-type 2 clinical trials. Expert Opin on Investig Drugs 2019; 28:811-820 [Impact factor: 5.081]

11.3. Scientific Conferences Attended

[1] 47th Annual Meeting of Hungarian Medical Association of America, Sarasota, Florida, USA, October 24 - 31, 2015. Poster Presentation

[2] Experimental Biology, San Diego, CA, USA, April 1-6, 2016, Poster Presentation

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