Gut region-specific alterations of the endogenous heme oxygenase system and pro-inflammatory cytokines in the enteric neurons of streptozotocin-induced diabetic rat model

PhD dissertation

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Contents

Abbreviations

3-NP	3-nitropropionic acid
AGEs	Advanced glycation end products
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF	Brain-derived neurotropic factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ENS	Enteric nervous system
GDNF	Glial-derived neurotrophic factor
GI	Gastrointestinal
GluR	Glutamate receptor
GP130	Glycoprotein 130
H_2O_2	Hydrogen peroxide
HbA1C	Glycated hemoglobin
HO	Heme oxygenase
HuC/D	Human neuronal protein
IL1	Interleukin 1
IL1α	Interleukin 1 alpha
IL1β	Interleukin 1 beta
IL1RI	Interleukin 1 receptor type I
IL6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IR	Immunoreactive
JAK	Janus kinase

LPS	Lipopolysaccharide
MnSOD	Manganese superoxide dismutase
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NADPH-d	Nicotinamide adenine dinucleotide phosphate-diaphorase
NANC	Non-adrenergic non-cholinergic
NGF	Nerve growth factor
NMDA	N-Methyl-D-aspartic acid
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOD	Non-obese diabetic
NOS	Nitric oxide synthase
Nrf2	Nuclear factor-like 2
OH^-	Hydroxyl group
$ONOO^-$	Peroxynitrite
OsO_4	Osmium tetroxide
P2X7	Purinoceptor 7
PB	Phosphate buffer
PI3	Phosphoinositide 3-kinase
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
SIL6R	Soluble interleukin 6 receptor
STAT3	Signal transducers and activators of transcription
STZ	Streptozotocin

T1DM	Type 1 diabetes mellitus
TBS	Tris-buffered saline
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF	Tumour necrosis factor
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TNFα	Tumor necrosis factor alpha
VIP	Vasoactive intestinal polypeptide

1. Introduction

The incidence of metabolic disorders like diabetes, cardiovascular diseases, and obesity has been increased considerably in recent decades[1]. Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease characterized by increased blood glucose levels (hyperglycaemia), which is mainly due to the destruction in insulin-secreting pancreatic islet β-cells. Hyperglycaemia is believed to be the most important factor in the pathogenesis of diabetic complications[2]. Several studies have reported that elevated blood glucose level induces alterations in different cell types (endothelial cells, retina, neurons), tissues, and organ systems that lead to the pathological conditions including endothelial dysfunction, retinopathy and enteric neuropathy[3-6].

1.1 Enteric nervous system

In mammals the enteric nervous system (ENS) structurally arranged into two major plexuses, myenteric (Auerbach's) and submucous (Meissner's) plexus which are interconnected by a network of neurons and glial cells (Figure 1). These plexuses contain the neural components of local reflex arcs, like primary afferent sensory neurons, interneurons and motor neurons[7].

The total number of enteric neurons approximately equal to the number of neurons in the spinal cord in the same species, in humans it is between 400-600 million[8]. The enteric neurons can be identified according to their location, neurochemistry, shape, projections, proportions, connections, and function. After intensive research from several laboratories over the past three decades, a full description of all classes of enteric neurons has been achieved in the guinea pig and mouse intestine[9-11]. Enteric neurons release primary transmitters (nitric oxide (NO), acetylcholine) and secondary neurotransmitters or modulators (serotonin or tachykinin)[8].

Within the myenteric plexus, the neural circuitry is predominantly regulates the contractile activities of the external musculature, whereas motor neurons of the submucous plexus regulate the secretomotor and vasomotor activities of the mucosa[8].

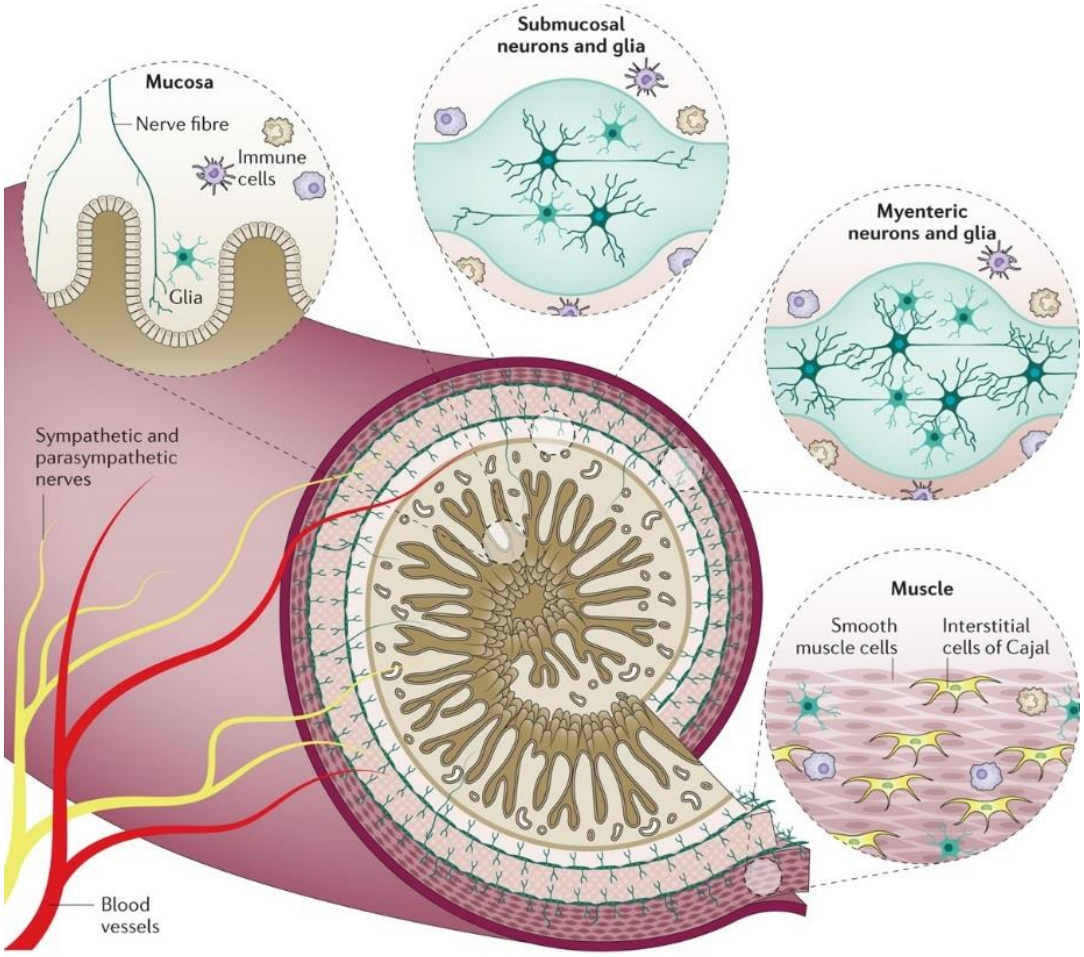


Figure 1. Schematic overview of key enteric nervous system components that control gastrointestinal function[12]

Therefore, any dysregulation in ENS can lead to gastrointestinal (GI) complications such as gastroparesis, motility disorders, delayed gastric emptying etc. It is reported that the patients with T1DM are often accompanied with GI motility symptoms[13] (Figure 2); and it is also well documented in diabetic animal models[14-16]. One of the main causes of this motility disorders are due to the altered expression of neuronal NO synthase (nNOS) in the GI tract. Moreover, there is evidence that nNOS neurons are highly affected in the GI tract of the streptozotocin (STZ)-induced diabetic rats. Reduced expression of nNOS or impairment of nNOS pathway have been reported in the myenteric plexus of stomach[17-19], duodenum[18,20,21], ileum[22] and colon[3,23].

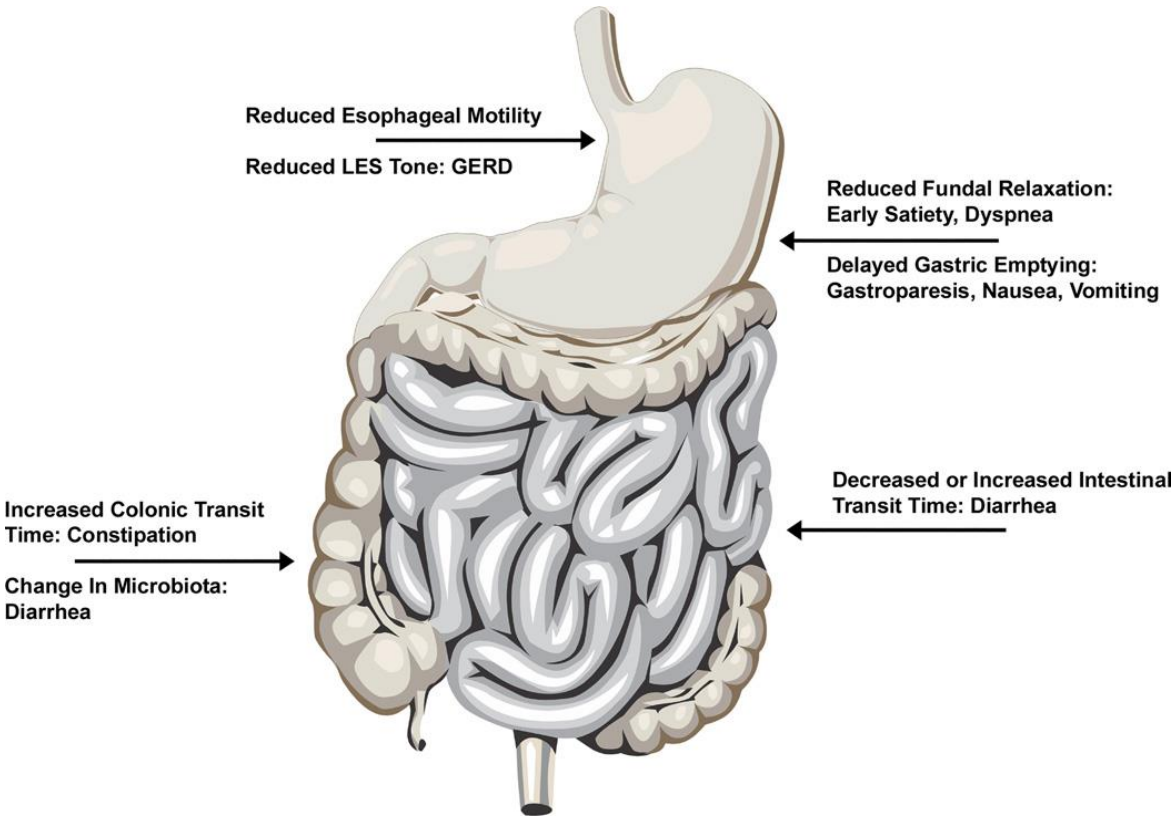


Figure 2. Effects of diabetes on motility in various parts of gastrointestinal tract[24]
LES- Lower esophageal sphincter and GERD- gastroesophageal reflux disorder.

1.2 Role of nitrergic neurons in diabetes

In the ENS, NO plays a significant role as a non-adrenergic non-cholinergic (NANC) neurotransmitter in regulating the blood flow, sphincters, and motility of the gut[25].

There are three genetically different isoforms of NO synthase (NOS) that account for NO production: nNOS, endothelial NOS (eNOS) and inducible NOS (iNOS). Of the three NOS isoforms, nNOS constitutes the predominant source of NO in neurons, while eNOS is the predominant source in the endothelium[25,26].

The nitrergic neurons in the myenteric plexus are type of inhibitory muscle motor neurons and descending interneurons[6,11]. Several studies have reported that the damage of myenteric nitrergic neurons are involved in the development of neuropathy in the digestive tract especially during diabetes[17-19,24,25,27], chronic ethanol consumption[28] and inflammation[29].

It is also reported that jejunum of spontaneously diabetic rats was characterised with myenteric nitrergic neuropathy by showing selective nitrergic motor dysfunction[18].

It is well documented that the anatomical, functional, and pathological regionality of the gastrointestinal tract develops under strict genetic control[30] which itself result in the unique susceptibility of the neurons to pathological conditions in different intestinal segments. According to our previous results, the nitrergic neurons located in different gut segments displayed different susceptibilities to diabetic and insulin treatment[3].

These findings were well correlated with the Cellek et al.[19] reported that streptozotocin-induced diabetic rats undergo a selective degenerative process in two phases. In the first phase, nitrergic nerve fibers lose some of their nNOS content and function. In the second phase, nitrergic degeneration takes place in the cell bodies in the ganglia, leading to complete loss of nitrergic function. The changes in the first phase are reversible with insulin replacement; however, the neurodegeneration in the second phase is irreversible.

Several studies also reported that nitrergic neuropathy appears to be more pronounced in the colon compared with the proximal gut[31-33]. The strict regionality of pathological processes showed more attention to the importance of the molecular differences in the neuronal microenvironment along the GI tract. Since the myenteric ganglia are not vascularized capillaries adjacent to them must be responsible for providing the ganglionic microenvironment, including the proper oxidative circumstances[6].

1.3 Oxidatives stress in diabetes

Numerous evidences have indicated that hyperglycaemia is a prime factor in the aetiology of oxidative stress which contributes to the pathogenesis of diabetic complications[34].

Free radical formation in diabetes by non-enzymatic glycation of proteins, glucose oxidation and increased lipid peroxidation leads to damage of enzymes, cellular machinery and also increased insulin resistance due to oxidative stress. In diabetes, main sources of oxidative stress are mitochondria. During oxidative metabolism in mitochondria, a component of the utilized oxygen is reduced to water, and the remaining oxygen is transformed to oxygen free radical ($O^{\cdot}$) which is an important reactive oxygen species (ROS) that is converted to other ROS such as peroxynitrite ($ONOO^{-}$), hydroxyl group (OH^{-}) and hydrogen peroxide (H_2O_2). Insulin signaling is modulated by reactive oxygen and nitrogen species (RONS) by two ways. On one side, in response to insulin, the RONS are produced to exert its full physiological function and on the other side, the RONS have

got negative regulation on insulin signaling, interpreting them to develop insulin resistance which is a risk factor for type 2 diabetes[34-35].

1.4 Protective role of endogenous heme oxygenase enzymes

To date, only few studies have been documented on the oxidative stress in the myenteric neurons during T1DM. It has been reported that the loss of enteric neurons is mainly due to increased oxidative stress and apoptosis in the colon of diabetic patients[13]. In addition, it is also reported that different subpopulation of myenteric neurons showed selective response to oxidative stress and diabetic stimuli[36]. Several studies have indicated that not only increased production of free radicals, but also the depletion of endogenous antioxidant system plays an important role in the pathogenesis of diabetes[37]. Among the endogenous antioxidant enzymes, heme oxygenase (HO) as a stress response protein and critical mediator of cellular homeostasis plays a protective role against enhanced hyperglycaemia-mediated oxidative stress and inflammatory responses.

HO, a rate limiting enzyme in the heme catabolism which produces biologically active carbon monoxide, iron and bilirubin (Figure 3). Three main HO isoforms have been reported and which are coded by different genes with different expression pattern mainly reported in tissue and cells[38,39].

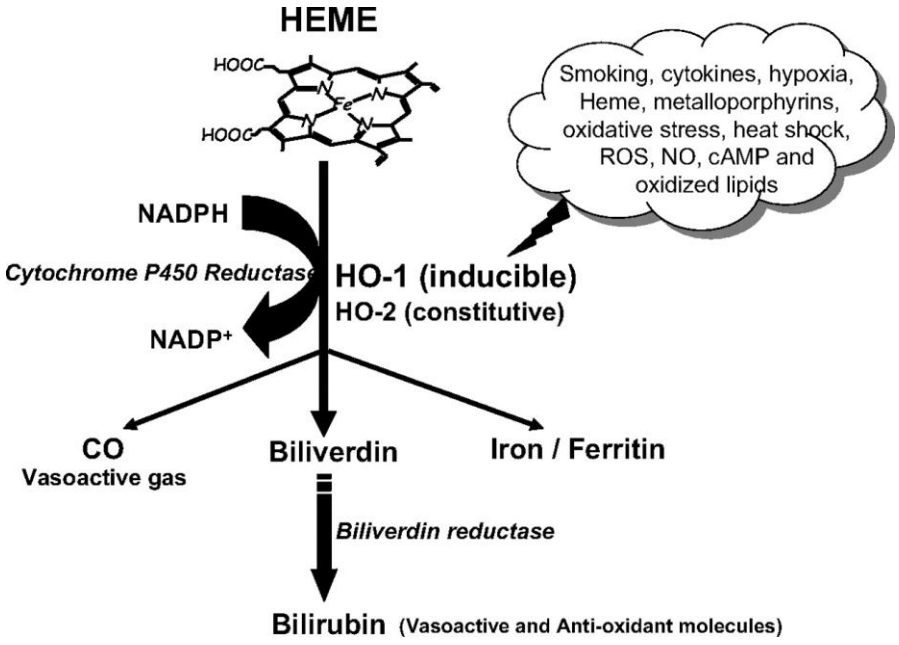


Figure 3. Schematic representation of the heme degradative pathway[40]
ROS- Reactive oxygen species, NO- nitric oxide, cAMP- cyclic adenosine monophosphate, $NADP^+$- nicotinamide adenine dinucleotide phosphate and CO- carbon monoxide.

HO1 a stress-inducible protein and expressed relatively low level in most of the tissues[41], whereas HO2 is thought to be a constitutive enzyme, highly expressed in the neuronal tissues acts as a critical mediator of cellular homeostasis[42,43]. HO3 with poor heme catalytic activity are found in rat brain, whereas no occurrence was observed in humans[44,45].

The induction of HO1 have been reported to play a protective role against oxidative stress[41,46], ischaemia-reperfusion[47], hypoxia[48], ROS[49] and inflammation[50] (Figure 4).

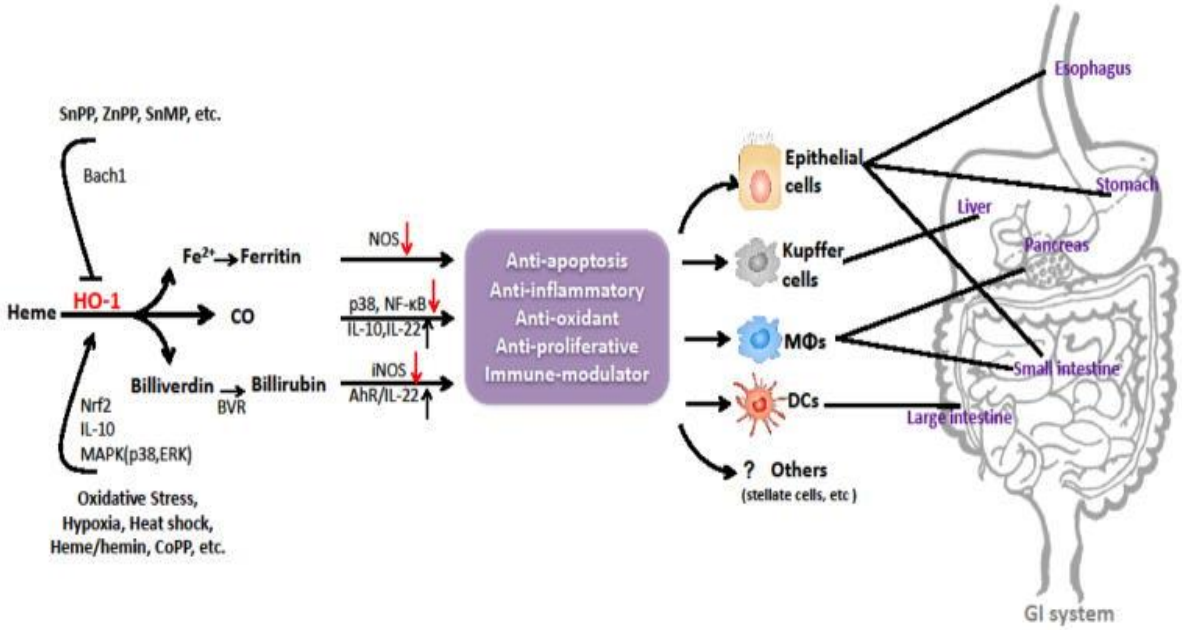


Figure 4. Hemeoxygenase1 (HO1) induction has a protective role in the gastrointestinal (GI) system[48]. Up-regulation of HO-1 and/or its downstream effectors in different cells and tissues is associated with protection against oxidative stress and tissue injury. AhR- aryl hydrocarbon receptor, Bach1- heme binding protein, BVR- biliverdin reductase, CoPP- cobalt protoporphyrin, DC- dendritic cells, HO-1- hemeoxygenase-1, iNOS- inducible nitric oxide synthase, Mϕs- macrophages, NOS- nitric oxide synthase, Nrf2- nuclear erythroid 2-related factor-2, SnMP- tin mesoporphyrin, SnPP- tin protoporphyrin, ZnPP- zinc protoporphyrin.

The HO1 induction showed rapid, protective antioxidant response in rhabdomyolysis of rats[51]. It has been reported that induction of HO1 protects interstitial cells of Cajal from oxidative stress by restoring the nNOS expression in diabetic gastroparesis in mice[52]. There is also evidence that HO1 induction promotes neuron to survive during spinal cord injury in rats[53]. HO1 induction also played a protective role in several diabetic complications[54-56] (Figure 5).

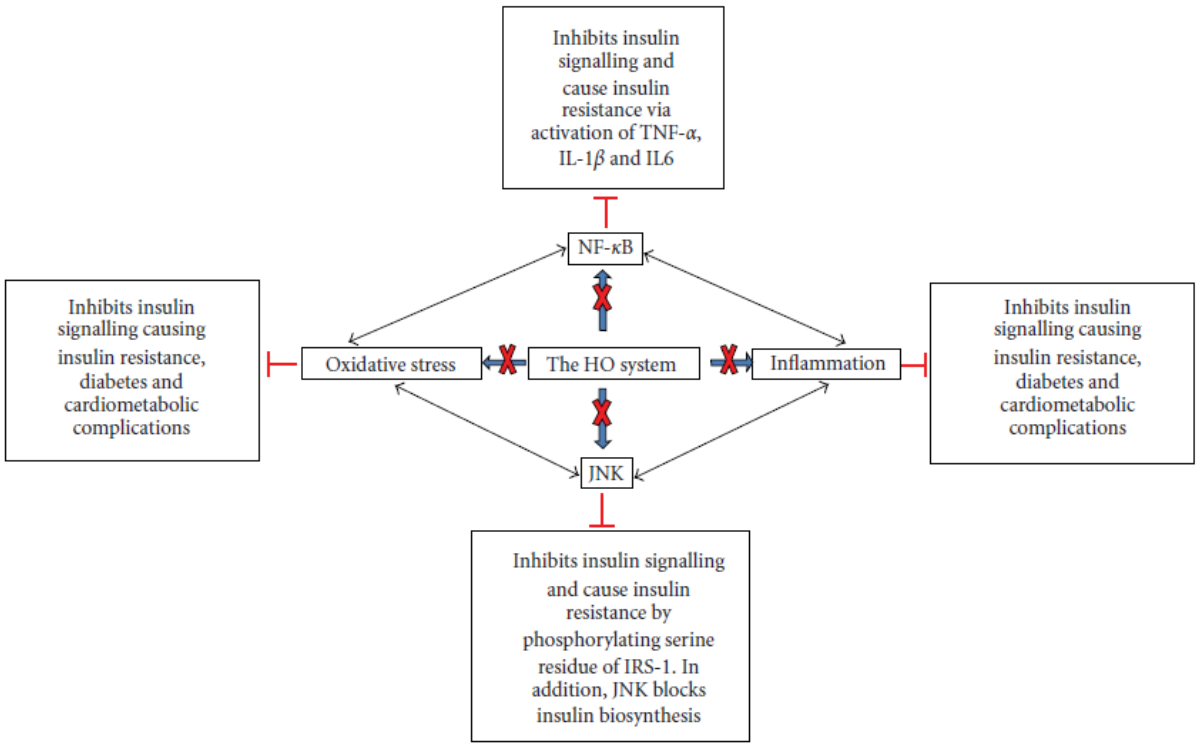


Figure 5. Schematic representation illustrating the protective role of the heme oxygenase (HO) system in glucose metabolism[57]. Inflammatory and oxidative mediators like NF-κB- nuclear-factor kappaB, JNK- c-Jun-N-terminal kinase, TNF-α- tumour necrosis factor alpha, IL-1β- interleukin-1 beta and IL6- interleukin 6 are amongst the pathophysiological factors that impair insulin signalling. Insulin receptor substrate-1 (IRS-1)

In addition, in rat ileum the antioxidant HO2 protects those NOS-containing neurons from oxidative stress in which it is co-localized[22]. It is also evident that HO2 played a neuroprotective role against intracerebral haemorrhage[58]. Due to the beneficent effects of the HO system[59], these endogenous antioxidants can be the most important players in the prevention of oxidative injury and diabetic GI complications. Moreover, treatment with antioxidants (L-glutamine and vitamin E) minimizes or prevents the development of widespread complications in diabetic patients[60,61]. However, HO induction may not always be beneficial: it can act also as a pro-oxidant mechanism depending on the disease milieu[62,63] and the duration of HO stimulation might be a critical factor in triggering either the cytotoxic or the adaptive responses[64].

1.5 Dual role of pro-inflammatory cytokines

Tumor necrosis factor alpha

Besides the alterations of endogenous antioxidant system, the pro-inflammatory cytokines are also considered to be important cellular mediators in the ENS. Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukins are mainly produced by immune cells and are shown to have wide variety of biological functions via regulating immune response, haematopoiesis, and inflammation[65].

TNFα is a biologically active transmembrane soluble protein which is mainly expressed by immune cells, endothelial cells, fibroblasts, neurons, astrocytes, and microglial cells; its expression is strongly upregulated by certain pro-inflammatory factors such as lipopolysaccharide (LPS) or other bacterial products and interleukin 1 beta (IL1β) (Figure 6).

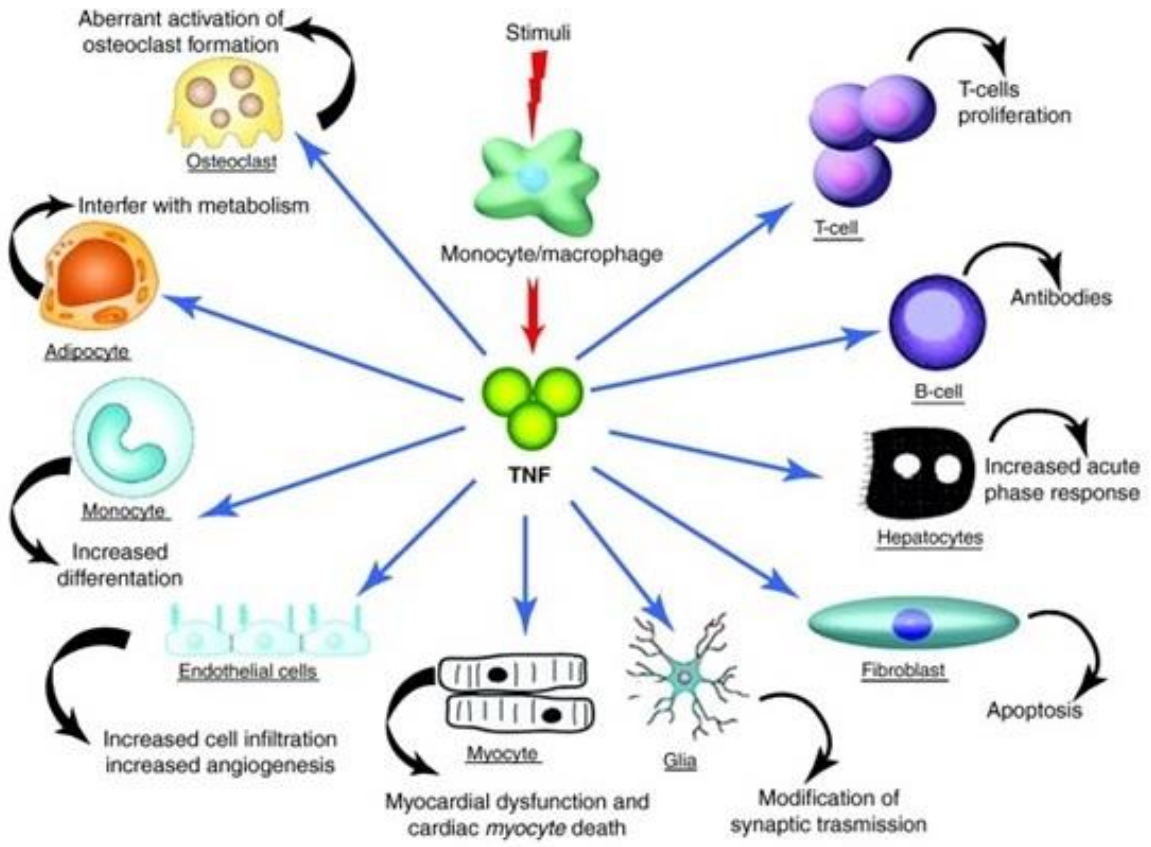


Figure 6. Cellular targets of tumour necrosis factor (TNF)[65]
TNF acts on a wide variety of cell types to mediate their biologic functions.

TNFα exhibits its biological action through cell surface receptors: tumor necrosis factor receptor 1 (TNFR1) and tumor necrosis factor receptor 2 (TNFR2)[67-70] Its pleiotropic functions are indispensable in the differentiation, growth and apoptosis of both immune and nonimmune cells[66,71] (Figure 7).

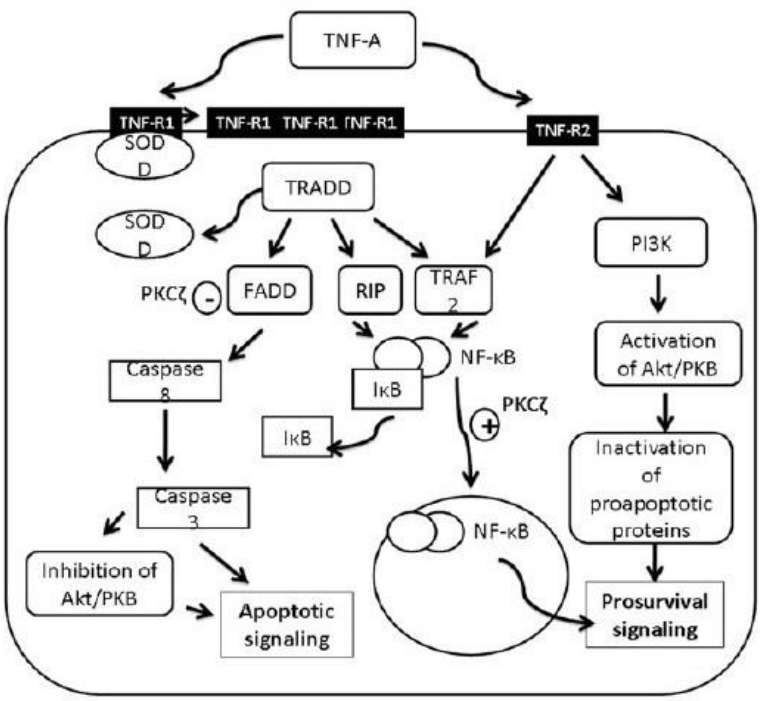


Figure 7. Molecular mechanisms in TNFα-induced prosurvival and apoptotic signaling[72]
SODD- Silencer of Death Domain, TRADD- TNF Receptor-Associated Death Domain, FADD- Fas-Associated Death Domain, RIP- Receptor Interacting Protein, NF-kB- nuclear factor kappa B, TRAF-2- TNF-R Asscoiated Factor 2, IkB- inhibitory protein, PKCz- protein kinase C zeta, Akt/PKB- Akt/protein kinase B

It is evident that elevated level of TNFα can induce apoptosis and it is believed to be associated with pathogenesis of inflammatory and autoimmune diseases[73]. In order to control the level of TNFα, several anti-TNF therapies have been introduced and clinically proved and successfully used to treat autoimmune diseases such as rheumatoid arthritis, and inflammatory bowel diseases[74,75]. However, this anti-TNF therapy was failed in treating neurodegenerative diseases such as multiple sclerosis and the negativity of results are mainly due to the pleiotropic action of TNFα including pro- and anti-inflammatory function and other immune regulatory as well as regenerative activities[74,76].

Several *in vivo* and *in vitro* study have reported that TNFα plays multiple roles in disease conditions. In non-obese diabetic (NOD) mice model, the treatment with TNFα at neonatal and 2 weeks-age mice showed same effect, whereas at the age of 4 weeks it delayed the onset of disease. Therefore, TNFα is highly dependent on age and particularly at the early age it is shown to play critical role in the development of autoimmunity towards beta-islet cells[76].

There is evidence that TNFα plays a dual role in T1DM. In islet cells during earlier stage of disease, TNFα expression was shown to enhance the inflammation at initial phase, whereas in later stage it abrogates the diabetes thereby showing a protective role in a time dependent manner[77]. *In vivo* treatment with TNFα reduces the lymphocytic infiltration and suppressed the induction of diabetes in adoptive transfer of lymphocytes. Thus, it protected the NOD mice from the disease progression of diabetes and insulitis[78]. Similarly, in NOD model, locally produced TNFα protected the autoimmune diabetes by preventing the development of auto-reactive islet-specific T cells[79]. Moreover, expression of TNFα was reported to be region-specific in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity. In this study, TNFα receptors deficient mice was induced by MPTP resulting aggravated neuronal damage in the hippocampus, while in the striatum MPTP was incapable to cause neuronal damage[80]. Therefore, TNFα expression is believed to be region-specific and time-dependent to play either beneficial or harmful effect on neurons.

TNFα not only plays a dual role in autoimmune diseases but also in neurodegenerative diseases. Other than the deleterious effect to neurons, it has been reported that TNFα acts as a neuroprotective agent for the neurons by preventing neurite outgrowth, axonal regeneration, and promoting regrowth of transected optic nerves[80,81]. These protective functions highlighting the pro-survival effect of TNFα in central nervous system pathology. Several evidences have reported that TNFα plays a crucial role in maintaining intracellular Ca^{2+} concentration resulting in reduction of neuronal injury against glucose deprivation, glutamate, N-Methyl-D-aspartic acid (NMDA) receptor or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) in cultured hippocampal neurons. Thus, TNFα acting as a cryoprotective agent to neurons[83-86].

Another important property of TNFα is that it exerts modulating effect on neuronal cell function by stimulating the nerve growth factor (NGF) in fibroblasts and glial cells. The study suggested that treatment with TNFα in primary astrocytes, upregulates glial-derived neurotrophic factor (GDNF) and brain-derived neurotropic factor (BDNF)[87,88]. It is

also evident that TNFα released endogenously in astrocytes by stimulation of LPS also induces the production of NGF and GDNF in astrocytes[89]. Similarly, TNFα and ILlβ were acted as a neurotrophic factor by promoting neurite growth for myenteric neurons in TNBS-colitis model[90]. Hence, TNFα indirectly regulates the various neurotrophic factors thereby playing an important role in the neuronal development.

TNFα plays a neuroprotective role in cultured neurons against an excitotoxic death mediated by glutamate receptor (GluR) agonist NMDA[91]. Moreover, treatment of cortical neuronal cultures with TNFα, IL1 (α/β) or IL6 showed concentration-dependent neuroprotective effect against an excitotoxic challenge with NMDA[92]. It is an evident that TNFα promotes neuronal survival and oligodendrocytes regeneration in *in vivo* models of retinal ischemia and neurotoxic insults[93,94]. TNFα protected the cortical neurons from glutamate-induced excitotoxicity in *in vitro* studies[95,96]. It has been reported that TNFα showed early protection against retinal ganglion cell loss[97]. Production and release of TNFα by purinoceptor7 (P2X7) receptor-activated microglia protects neurons against glutamate neurotoxicity[98].

Pre-treatment of neurons or astrocytes with TNF attenuated manganese superoxide dismutase (MnSOD) activity and also against 3-nitropropionic acid (3-NP) induced superoxide accumulation and preventing the loss of mitochondrial membrane potential[99]. Furthermore, TNFα have been found to modulate the inflammatory response including other factors such as IL1β, adhesion molecules, and metalloproteinases[100]. There is evidence that TNFα showed protective role on diabetes-induced vascular inflammation in STZ-induced mice[101].

Interleukin 1 beta

IL1β is a prototypic cytokine which plays a central role in coordinating host immune and pro-inflammatory responses. It is mainly involved in enhancing the production of immune-related molecules such as adrenocorticotropin, cytokines, and chemokines. IL1β are released by different cell types such as keratinocytes, fibroblasts, synoviocytes, endothelial, neuronal, immune cells (macrophages and mast cells) and glial cells (Schwann cells, microglia cells and astrocytes). Interaction of IL1β with the type 1 IL1 receptor (IL1RI) elicits a cascade of immune responses[102,103]. It is reported that IL1β has important homeostatic functions in the normal organism such as in the regulation of feeding, sleep, and temperature. However, overproduction of IL1β is implicated in the

pathophysiological changes that occur during different disease states such as neuropathic pain, inflammatory bowel disease, vascular disease, multiple sclerosis, and Alzheimer's disease[104].

A number of clinical studies have reported increased IL1β secretion from colonic tissues and macrophages of inflammatory bowel disease patients. It is reported that IL1β mRNA levels were highest in active ulcerative colitis and even the noninflammed gut segment of inflammatory bowel disease patients. Furthermore, the clinical efficacy with therapies targeting IL1β has demonstrated a critical role of IL1 (α/β) in certain autoimmune diseases[105,106]. It is evident that IL1β-induced increase in intestinal epithelial tight junction permeability has been postulated to be an important mechanism contributing to intestinal inflammation of Crohn's disease and other inflammatory conditions of the gut[107].

Evidence have reported the possible role of IL1β in the development of diabetic retinopathy[108]. It is reported that elevated serum level of IL1β was observed during early phase of T1DM. Thus, it is acting as indicator of an ongoing β-cell destruction in T1DM children[109]. Recently, it has been reported that IL1β-dependent pathway is impaired in T1DM which lead to enhanced neurite outgrowth through Janus kinase/signal transducers and activators of transcription (JAK/STAT3) modulation of mitochondrial bioenergetics in adult sensory neurons[110]. There is also evidence that inhibition of acetylcholine induced intestinal motility by IL1β in the experimentally induced colitis rats[111]. Moreover, it is reported that IL1 (α/β) are important for the generation of early-phase protective immunity against mycobacterial infection in mice[112].

Interleukin 6

IL6, a pleiotropic cytokine, based on their biological role is named as ciliary neurotrophic factor, leukemia inhibitory factor, oncostatin M, cardiotrophin-1, and neurotrophin-1B-cell stimulating factor[113]. It is secreted by both immune cells (macrophage, T cells, B cells), and non-immune cells (keratinocytes, endothelial cells, fibroblasts, astrocytes, mesangial, intestinal epithelial cells, glial cells and neurons) and by various stimuli such as viruses, bacteria, LPS[65].

Like TNFα, IL6 mediates its pleiotropic action through its receptor complex as IL6Rα and glycoprotein 130 (gp130), although it also mediated by soluble IL6 receptor. It plays a tremendous role in the acute phase response, tissue regeneration, inflammation and

pathogen defence mechanism (Figure 8). Its expression is strongly upregulated in inflammatory diseases as well as neurological diseases[114,115]. However, its expression was also shown to be protective in many diseases conditions such as diabetes-related neuropathy[113], ischemia-reperfusion injury[116], and oxidative stress[117].

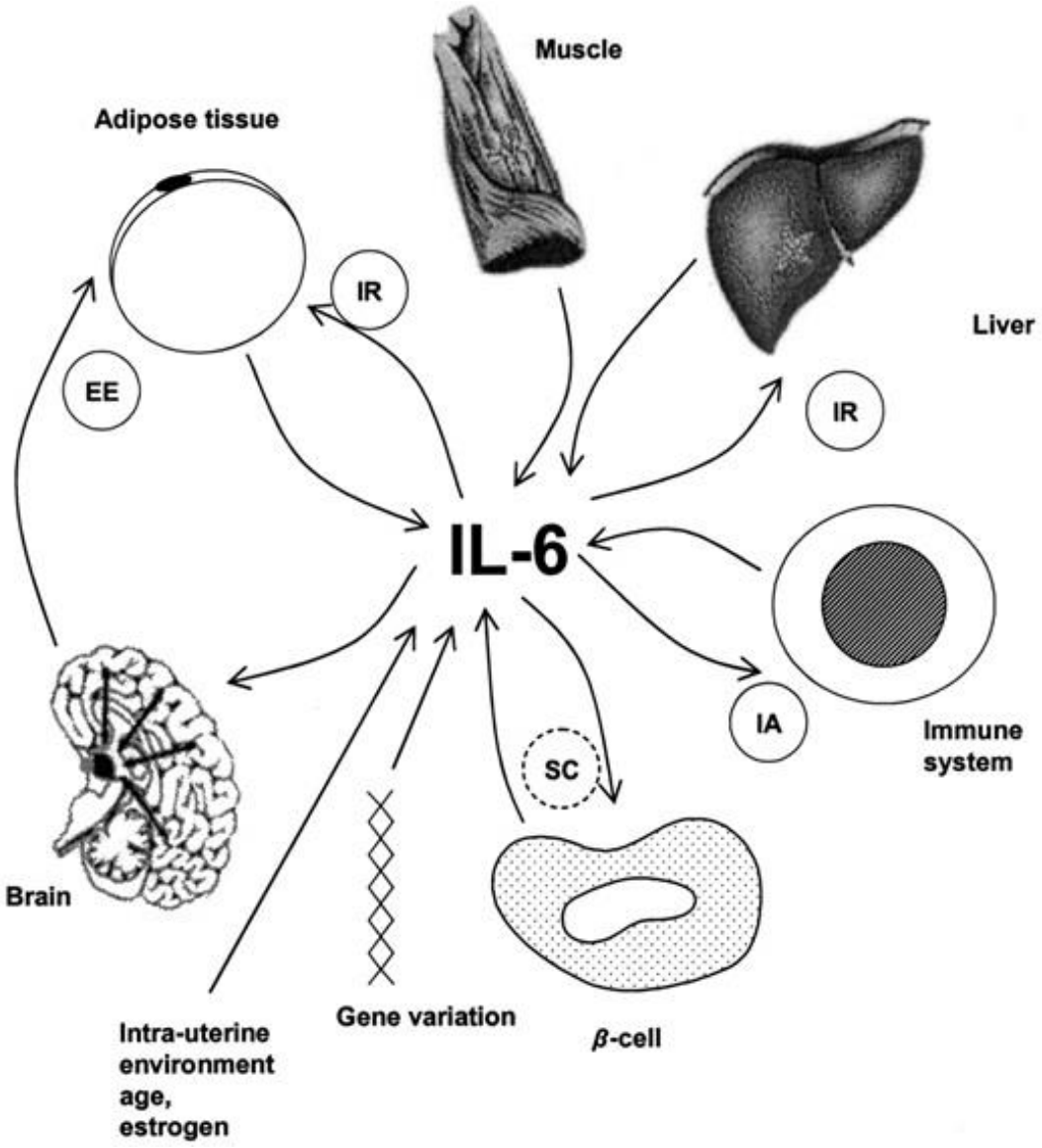


Figure 8. Model of IL-6 actions of potential relevance for the pathogenesis of type 1 and type 2 diabetes[118]. IL-6 induces insulin resistance (IR) in adipose tissue and liver and may synergize with proinflammatory cytokines to produce β-cell damage. IL-6 also regulates energy expenditure probably by the effect on brown adipose tissue via effects on the central nervous system. EE- energy expenditure, IA- immune activation, IR- insulin resistance, SC- synergy with proinflammatory cytokines.

Several *in vitro* studies have been reported that IL6 functioning as neurotrophic factor for survival of cortical, mesencephalic and sensory neurons[119,120]. Moreover, the study demonstrated that treatment with IL6/sIL6R showed neurite outgrowth and neuronal survival in the myenteric plexus of rats[121]. Conversely, IL6 deficient mice showed alteration in the development of peripheral sensory functions and regeneration. Thus IL6 is essential for the developmental function of peripheral neurons[122]. In addition, IL6/sIL6R are shown to be involved in the peripheral nerve regeneration[123]. Genetically deficient IL6 mice demonstrated the reduced neuroglial activation in a traumatic model of central nervous system[124]. However, upregulation of IL6 has been reported to cause neurodegenerative disorders in brain[125].

Pretreatment with IL6 protects various types of neurons of central nervous system against intoxication with glutamate, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPP+) neurotoxicity[126-128]. There is also evidence that administration of kainic acid-induced neurotoxicity in IL6 deficient mice shown to reduce brain inflammatory response and increases oxidative stress in hippocampal tissue[117]. In addition, the study demonstrated that IL6 deficient mice treated with trimethyltin showed neuroprotective effect by the activation of Nrf2-dependent glutathione homeostasis and PI3/akt signalling mechanism[129].

There is also a few evidence from *in vivo* studies which showed that IL6 protects neuronal injury and neuron loss from ischemia and axotomy-induced motoneuron loss[130,131]. Another study demonstrated that chronic intraperitoneal or subcutaneous administration of IL6 is neuroprotective against development of diabetes-related neuropathy[113]. It has been reported that IL6, shown to play various roles as a neuroprotective and potent nerve growth factor for mature retinal glial cells[132].

Unlike TNFα, IL6 is shown to be an *in vivo* inhibitor of apoptosis in the enterocytes and intestinal tract. In this study, IL6-treated mice were observed with less intestinal injury and improved barrier function in the gut. Therefore, IL6 plays regulatory role in the GI disorders associated with apoptosis and injury[133]. In addition, pre-treatment with IL6 in mice after induction of hypothermia were shown to protect the intestine from injury, reduced the permeability of intestinal barrier. Therefore, endogenous expression of IL6 at early stage suggested to play a protective role in the development of organ damage and inflammation[115].

Alternatively, the transplant studies reported that IL6 infusion before 2 hours of transplant showed effective prevention against intestine ischemia/reperfusion injury[116]. In IL6 deficient mice treated with IL6 improved dextran sodium sulfate-induced colitis[134]. However, it has been reported that increased production of TNFα, IL1β, and IL6 were observed in intestinal biopsies of Crohn's disease patients[135]. Similarly, in transplant studies, neural stem cells preconditioned with IL6 by inducing ischemia/reperfusion tolerate oxidative stress and induce angiogenesis. Hence it is believed to be involved in the cell survival mechanisms[136]. It has been demonstrated that ischemia-induced brain injury has been protected by endogenous IL6 against NMDA receptor-mediated excitotoxicity[137].

Recently, it has been reported that IL6 promotes axonal regrowth and enhances synapse formation after spinal cord injury[138]. IL6 has shown to possess homeostatic role that limits the obesity-associated insulin resistance and inflammation[139].

We have previously demonstrated, that nitrergic neurons which play a key role in gut motility are involved in GI complications[3]. These results highlight the importance of the neuronal microenvironment along the GI tract in the pathogenesis of diabetic nitrergic neuropathy. In order to get a closer view, based on the molecular differences in the neuronal microenvironment, we have demonstrated that the mesenterial capillaries supplying the myenteric ganglia[140] and the faecal-associated microbiota[141] are also serious targets of diabetic injuries.

Recently, we have reported that increase of the hyperglycaemia-induced oxidative stress and decreased effectiveness of the endogenous antioxidant enzymes (superoxide dismutase, catalase, HO system) plays a major role in the initiation of diabetes-related neuronal damage[142]. However, there is a considerable gap still remains in understanding the neurochemical role of nitrergic neurons in response to endogenous antioxidant system and to date, only few studies have been documented on the oxidative stress in the myenteric and submucous neurons during T1DM.

2. Aims

In this study, we used a STZ-induced diabetic rat model which is suitable for investigating the effects of chronic hyperglycaemia and immediate insulin replacement in the ENS and its microenvironment along the GI tract.

Gut segments of duodenum, ileum and colon were processed for double-labelling fluorescent and post-embedding immunohistochemistry as well as enzyme-linked immunosorbent assay (ELISA) to determine the intestinal region-specific alterations in the expression of the endogenous antioxidant system and pro-inflammatory cytokines through the following questions:

HO system in the myenteric plexus

- Is there any gut segment-specific difference in the number of myenteric HO1- and HO2-immunoreactive (IR) neurons?
- Is there any effect of diabetes on the HO-nNOS co-localization in the myenteric plexus of the different intestinal segments?
- Is there any effect of diabetes on the serum and tissue level of HO1 and HO2?

HO system in the submucous plexus

- Is there any segment-specific effect of diabetes and immediate insulin treatment on the proportion of the nNOS, HO1- and HO2-IR submucous neurons?

Pro-inflammatory cytokines

- Is there any gut segment-specific difference in the expression of TNFα and IL6 in the myenteric ganglia and surrounding capillary endothelium of the control, diabetic and insulin-treated diabetic rats?
- Is there any effect of the diabetes and insulin-treatment on the tissue level of TNFα, IL1β and IL6 in the tissue homogenates containing myenteric or submucous plexus?

3. Materials and Methods

3.1 Animal model

Adult male Wistar rats (Crl:WI BR; Toxi-Coop Zrt.) weighing 210-260 g, kept on standard laboratory chow (Farmer-Mix Kft., Zsámbék) and with free access to drinking water were used throughout the experiments.

For the 10-week chronic hyperglycaemic study the rats were divided randomly into two groups: STZ-induced diabetics (n=6) and sex- and age-matched controls (n=10). Besides these two groups, for the investigation of the HO system in the submucous plexus and the expression of the pro-inflammatory cytokines, a third group, an insulin-treated diabetic group was also used (n=4).

The hyperglycaemia was induced by a single intraperitoneal injection of STZ (Sigma, USA) at a dose of 60 mg/body weight kg. After 48 hours of STZ-treatment, blood was taken from the tail vein and blood glucose level were measured by the glucose oxidase method, using a portable blood glucose monitoring device (ACCU-CHEK Active, Roche Diabetes Care GmbH, Germany). The animals were considered diabetic if the non-fasting blood glucose concentration was higher than 18 mM. Simultaneously one group of hyperglycaemic rats received a subcutaneous injection of insulin (Humulin M3, Lilly, USA) each morning and afternoon (2-2 IU)[3].

The animal health condition was monitored daily during the experimental period. The blood glucose level and the weight of each animal were measured daily.

The experiments were performed with strict adherence to the National Institutes of Health (Bethesda, MD, USA) guidelines and the EU directive 2010/63/EU for the protection of animals used for scientific purposes. The study was approved by the National Scientific Ethical Committee on Animal Experimentation (National Competent Authority), with the license number XX/1487/2014. The rats were kept in the animal care facility of the Department of Physiology, Anatomy and Neuroscience, University of Szeged in a 12/12-h day/night cycle under standard air temperature and humidity conditions. Food and water were provided ad libitum.

3.2 Blood collection and tissue handling

Ten weeks after the onset of diabetes, blood samples were collected from the tail vein of each animal. The tail was immersed in warm water (20-30 °C) for 40-50 sec to dilate blood vessels and to remove the dirt or faeces. Then the tail was dried with a paper

towel to identify the clear veins. The 22 G butterfly needle tip was inserted on one of the lateral tail veins (around 5 mm) at a position approximately 2-3 cm away from the tip of the tail at angle of approximately 20°. Then the blood was collected into the vacuum collection tube (BD Vacutainer® SST™ II Advance) by inserting the rubber end of the butterfly needle into the vacuum blood collection tube. The collected blood samples were centrifuged for 10 minutes at 3200 rpm at 20 °C and supernatant was used for glycated haemoglobin (HbA1c) assay and ELISA.

After the blood collection, the animals of the chronic diabetic experiment were killed by cervical dislocation under chloral hydrate anaesthesia (375 mg/kg ip).

The gut segments of the control, STZ-induced diabetic and insulin-treated diabetic rats were dissected and rinsed in 0.05 M phosphate buffer (PB), pH 7.4. A 10 cm-long intestinal segments were taken from the duodenum (1 cm distal to the pylorus), the ileum (1 cm proximal to the ileo-caecal junction) and the proximal colon and processed for immunohistochemical studies and ELISA.

For double-labelling immunohistochemistry, 4 cm-long samples were cut along the mesentery, pinched flat and fixed overnight at 4 °C in 4% paraformaldehyde solution buffered with 0.1 M PB (pH 7.4). The samples were washed thoroughly with 0.05 M PB (pH 7.4) and whole-mount preparation of myenteric plexus were made after removing the mucosa, submucosa, and circular smooth layer of the gut wall[3]. To obtain the whole-mounts of the submucous plexus, the muscular layers of the gut wall were removed, and the mucosa was extracted by scrapping with a small spatula under an Olympus SD30 stereomicroscope. For ELISA, 3 cm-long samples were cut along the mesentery and pinched flat. The layer of mucosa and submucosa were removed without any fixation under stereomicroscope. Then the residual material was snap-frozen in liquid nitrogen and stored at -80 °C until use. For electron microscopic studies, a tube shaped gut segments (2-3 mm) were fixed overnight at 4 °C in 2% paraformaldehyde and 2% glutaraldehyde solution, buffered with 0.1 M PB (pH 7.4). The samples were then washed in 0.05 M PB and further fixed for 1 h in 1% OsO_4. After fixation, the gut segments were rinsed in 0.1 M PB, dehydrated in increasing alcohol concentrations (50, 70, 96% and absolute ethanol) and acetone, and embedded in Embed 812[140].

3.3 HbA1c assay

Serum level of HbA1c was analysed by standard clinical chemistry assay on an Automated Chemistry Analyzer (BiOLis 24i, Tokyo Boeki Machinery Ltd, Tokyo, Japan)[143].

3.4 Measurement of serum protein levels by ELISA

Serum levels of HO1 and HO2 were measured by means of quantitative ELISA according to the manufacturer's instructions (SunRed Biotechnology, Shanghai, China). In briefly, 40 µl of serum, 50 µl of standard diluent was added to HO1 or HO2-coated 96-well plates, followed by 10 µl of biotin-(HO)-antibody was added to the sample well. 50 µl of streptavidin-HRP was added to each well and incubated for 60 min at 37 °C. Once the well-plates were washed, immediately 50 µl of chromogen solutions (A and B) were added to each well and incubated for 10 min at 37 °C in the dark. After adding stop solution, optical density was measured at 450 nm (Benchmark Microplate Reader; Bio-Rad, Budapest, Hungary) and the concentrations were expressed as ng/ml.

3.5 Quantitative fluorescent immunohistochemistry

For double-labelling fluorescent immunohistochemistry all incubations were carried out at room temperature. All primary and secondary antibodies were diluted in PB containing 10% goat serum, 0.1% bovine serum albumin. Blocking nonspecific antibody staining, whole-mount preparations were placed for 30 min in PB containing 0.1% bovine serum albumin, 10% normal goat serum and 0.3% Triton X-100 before incubation in primary antibodies (Table 1).

Myenteric whole-mount preparations were immunostained overnight with antibodies against nNOS and HO1 or HO2, whereas the submucous whole-mount preparations with antibodies against nNOS and HuC/D, HO1 and peripherin as well as HO2 and peripherin. After washing in PB, whole-mounts were incubated with anti-rabbit Alexa Fluor 488 and anti-mouse CyTM3 secondary antibodies for 4 h (Table 2).

Table 1. Details of primary antibodies used for fluorescent immunohistochemistry

Primary antibody	Host species	Dilution	Manufacturer
anti-nNOS	rabbit	1:200	Cayman Chemical, USA
anti-HuC/D	mouse	1:50	Molecular Probes, Eugene, OR USA
anti-HO1	mouse	1:200	Novus Biologicals Europe, Abington, UK
anti-HO2	mouse	1:50	Santa Cruz Biotechnology, Inc., USA
anti-peripherin	rabbit	1:400	Millipore, Temecula, CA, USA

Table 2. Details of secondary antibodies used for fluorescent immunohistochemistry

Secondary antibody	Dilution	Manufacturer
anti-rabbit Alexa Fluor 488	1:200	Life Technologies Corporation, Molecular Probes, Inc., Eugene, OR USA
anti-mouse CyTM3	1:200	Jackson ImmunoResearch, Inc., Baltimore Pike, PA

All incubations were carried out at room temperature. Negative controls were performed by omitting the primary antibody, when no immunoreactivity was observed. Whole-mounts were mounted on slides in EverBriteTM Mounting Medium (Biotium, Inc., Hayward, CA, the United States of America), observed and photographed with an Olympus BX51 fluorescent microscope equipped with an Olympus DP70 camera.

The proportions of neurons in which immunoreactivity was colocalized were determined by examining fluorescently labeled, double-stained preparations. Neurons were first located by the presence of a fluorophore that labeled one antigen, and then the illumination was switched to determine whether the neuron was labeled for a second antigen located with a fluorophore of a different colour. In this way, the proportions of neurons labeled for pairs of antigens were determined.

From the myenteric plexus, 50 ganglia were investigated from each intestinal segment per experimental groups and percentage of ganglia containing HO-IR or nNOS-HO-IR neurons or none of these were also determined. We counted the number of nNOS-IR, HO1- or HO2-IR neurons and those myenteric neurons in which the two markers were

co-localized (per ganglia). From the submucous plexus, 100 ganglia were taken from each intestinal segment in each experimental group and we counted the nNOS-, HO1- and HO2-IR neurons and the HuC/D- and peripherin-IR neurons to yield the total number of submucous neurons. We determined the nNOS-, HO1- and HO2-IR neurons as a percentage of the total number of neurons. Data were collected from preparations obtained from at least three animals.

3.6 Post-embedding immunohistochemistry and transmission electron microscopy

The Embed 812 blocks were used to prepare semithin (0.7 μm) sections, which were stained with toluidine blue to select area of interest. For ultrathin (70 nm) sectioning, three Embed 812 blocks were used from all three intestinal segments and each groups. Then the sections were mounted on nickel grids. The ultrathin sections were processed for TNFα and IL6 gold-labelling post-embedding immunohistochemistry. Ultrathin sections from each block were sequentially incubated in 1% bovine serum albumin in TBS for 30 minutes, then incubated overnight in the primary antibodies (Table 3), followed by secondary antibodies (Table 4) for 3 hrs, with extensive TBS washing in-between. All steps were performed at room temperature. The specificity of the immunoreaction was assessed in all cases by omitting the primary antibodies in the labelling protocol and incubating the grids in a suspension of protein A-gold only.

Table 3. Details of primary antibodies used for post-embedding immunohistochemistry

Primary antibody	Host species	Dilution	Manufacturer
anti-TNFα	rabbit polyclonal IgG	1:25	Abcam, UK
anti-IL6	mouse polyclonal IgG	1:25	Abcam, UK

Three grids per block were stained with uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Merck) and were examined and photographed with a JEOL JEM 1400 transmission electron microscope (Bódi et al. 2012). Counting was performed on digital photographs of 5 myenteric ganglia and 5 mesenteric capillaries per intestinal segments in each experimental group at a magnification of 20000×. The total number of gold particles per unit area was counted in the entire profile of the myenteric ganglia and the

endothelium of capillary in the vicinity of the myenteric plexus with the ImageJ software (Wayne Rasband; National Institutes of Health, USA).

Table 4. Details of secondary antibodies used for post-embedding immunohistochemistry

Secondary antibody	Dilution	Manufacturer
anti-rabbit 18 nm gold-conjugated IgG	1:20	Jackson Immunoresearch, Inc., Baltimore Pike, PA
anti-mouse 18 nm gold- conjugated IgG	1:20	Jackson ImmunoResearch, Inc., Baltimore Pike, PA

3.7 Measurement of tissue HO1, HO2, TNFα, IL1β and IL6 concentrations

3.7.1 Tissue homogenization

The intestinal tissue samples were frozen in liquid nitrogen, crushed into powder in a mortar and homogenized in 500 µl homogenizing buffer (100 µl Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO) in 20 ml 0.05 M PB). Tissue homogenates were centrifuged at 5000 rpm for 20 min at 4 °C and used to determine the total protein content.

3.7.2 Bradford protein micromethod for the determination of tissue total protein content

For the determination of total protein content of tissue homogenates, a commercial protein assay kit was used. Bradford reagent was added to each sample. After mixing and following 10 min incubation, the samples were assayed spectrophotometrically at 595 nm. Protein level was expressed as mg protein/ml.

3.7.3 Determination of antioxidant enzymes and pro-inflammatory cytokines by ELISA

The tissue homogenates obtained from the different intestinal segments were used to measure the levels of HO1, HO2, TNFα, IL1β and IL6 by means of quantitative ELISA according to the manufacturer's instructions (SunRed Biotechnology, Shanghai, China). Optical density was measured at 450 nm (Benchmark Microplate Reader; Bio-Rad, Budapest, Hungary). The tissue concentrations of HO1, HO2 were expressed as ng/mg protein, while TNFα, IL1β and IL6 were expressed as pg/mg protein.

3.8 Statistical Analysis

Statistical analysis was performed with one-way ANOVA and the Newman–Keuls test. All analysis was carried out with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). A probability of $P<0.05$ was set as the level of significance. All data were expressed as mean ± SEM.

4. RESULTS

4.1 Disease characteristics in diabetic rats

The diabetic rats were characterized by reduced body weight gain and increased blood glucose concentration as compared to the insulin-treated diabetics and age- and sex-matched controls (Table 5). All the experimental groups showed significant weight gain throughout the study period. The final body weight was significantly decreased in the STZ-induced diabetics (382.7±3.53), whereas the insulin-treated diabetics did not show any change (481.5±13.4) compared to the control group (486±4.93).

After STZ treatment, the blood glucose concentration was significantly increased in the STZ-induced diabetics and insulin-treated diabetics as compared to the initial blood glucose (Table 5). The blood glucose concentration of the STZ-induced diabetics showed 4-fold increase (23.31±0.53) and insulin-treated diabetics remained at 9.5 mM but still it was significantly higher, compared to the control group (6.3±0.13). The insulin-treated diabetic rats showed a significant decrease in blood glucose concentration compared to the STZ-induced diabetic group (Table 5).

Table 5. Body weight and blood glucose concentration of the experimental groups

Groups	Weight (g)		Blood glucose concentration (mM)	
	Initial	Final	Initial	Final (average)
Controls (n=10)	232.2±7.29	486±4.93****	7.08±0.22	6.3±0.13
Diabetics (n=6)	235.3±10.48	382.7±3.53****††††	6.6±0.1	23.31±0.53****††††
Insulin-treated diabetics (n=4)	251.5±4.35	481.5±13.4****^{0000}	6.65±0.18	9.48±0.14****††††^{0000}

Data are expressed as mean ± SEM. ****P<0.0001 vs. initial, ††††P<0.0001 vs. final controls, ^{0000}P<0.0001 vs. final diabetics.

4.2 Serum level of HbA1c

The serum level of HbA1c was 2-fold elevated (9.13±0.26 %) in the STZ-induced diabetics compared to the control group. The immediate insulin treatment restored the level of HbA1c to the control stage (Table 6).

Table 6. Serum HbA1c level in the different experimental groups

Parameter	Controls (n=5)	Diabetics (n=6)	Insulin-treated diabetics (n=4)
HbA1c (%)	3.93±0.05	9.13±0.26****	3.98±0.05^{0000}

Data are expressed as mean ± SEM. ****P<0.0001 (controls vs. diabetics), ^{0000}P<0.0001 (diabetics vs. insulin-treated diabetics).

4.3 Serum levels of HO1 and HO2

In the diabetic rats, HO1 (5.90±1.09 ng/ml) and HO2 concentration (2.55±0.48 ng/ml) were decreased, whereas the significant differences were only seen in the HO1 as compared to the control group (5.90 vs. 9.65±0.50 ng/ml). In the insulin-treated diabetics, concentration of HO1 and HO2 were unchanged as compared to the control group (Figure 9A,B). However, HO1 showed significant differences between the insulin-treated diabetics and diabetic group.

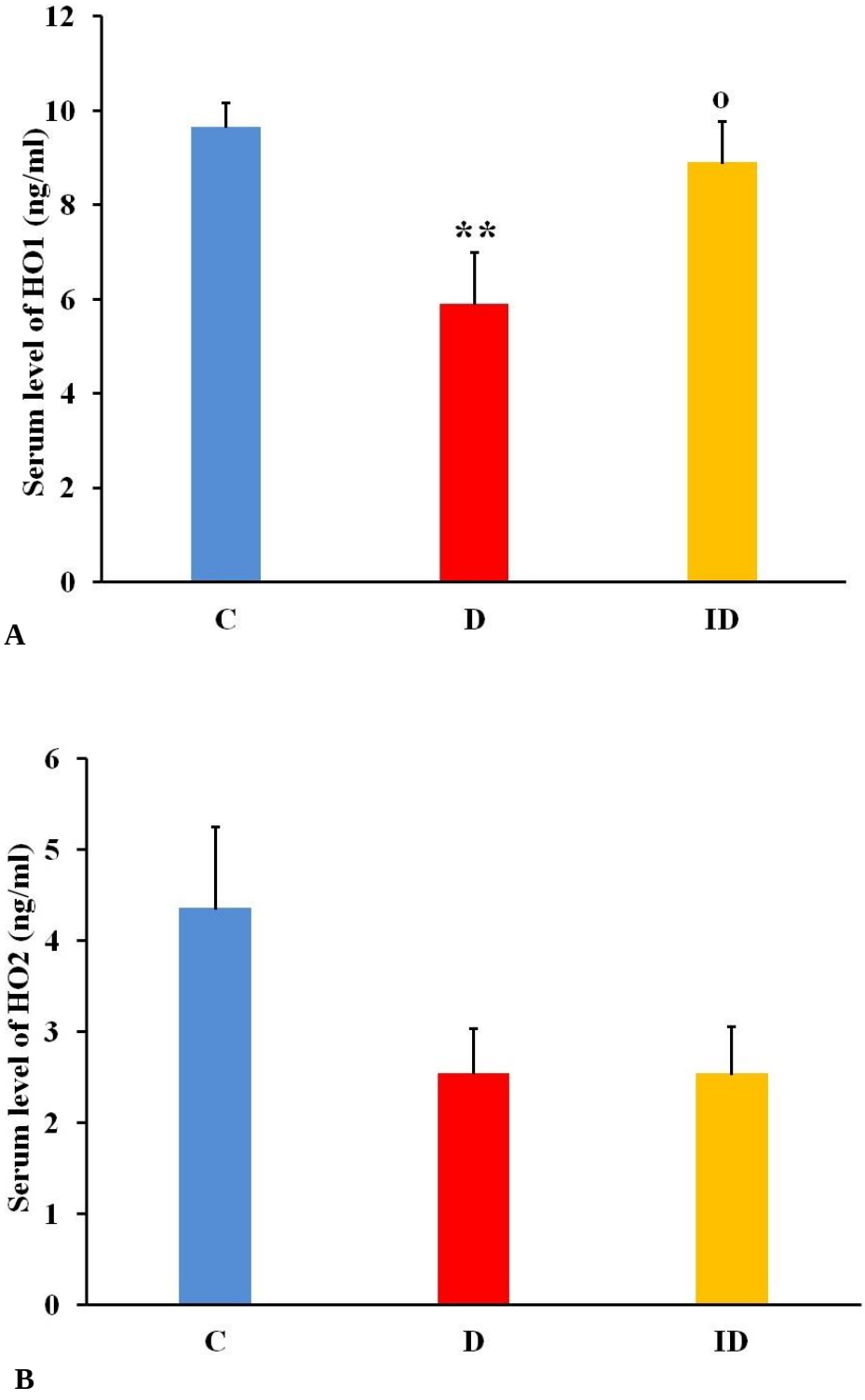
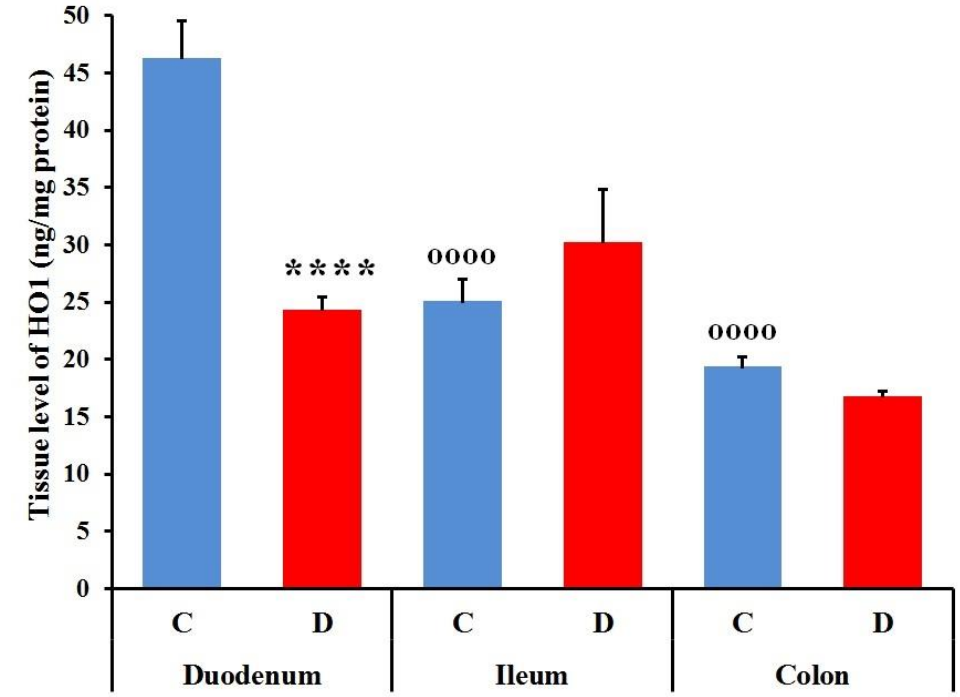


Figure 9. The serum level of HO1 (A) and HO2 (B) in the experimental groups. Data are expressed as mean ± SEM. **P<0.01 (controls vs. diabetics), °P<0.05 (diabetics vs. insulin-treated diabetics). C - controls; D - diabetics; ID - insulin-treated diabetics.

4.4 Tissue levels of HO1 and HO2

In the tissue homogenates including the smooth muscle layers of the intestinal wall and the myenteric plexus, the expressional patterns of HO1 and HO2 were strictly region-dependent in the control animals. The highest concentrations of HO enzymes were detected in the tissue homogenates of control duodenum with 46.35±3.22 ng/mg protein in the case of HO1 and 42.72±3.53 ng/mg protein in the case of HO2 (Figure 10A,B). In the ileum and colon of controls, the tissue level of HO1 and HO2 was significantly lower than in the duodenum. In the diabetics, the duodenum was the only gut segment where the tissue levels of HO1 and HO2 decreased strongly (24.35±1.12 ng/mg protein in the case of HO1 and 28.44±0.97 ng/mg protein in the case of HO2), whereas they remained unchanged in the ileum and colon.



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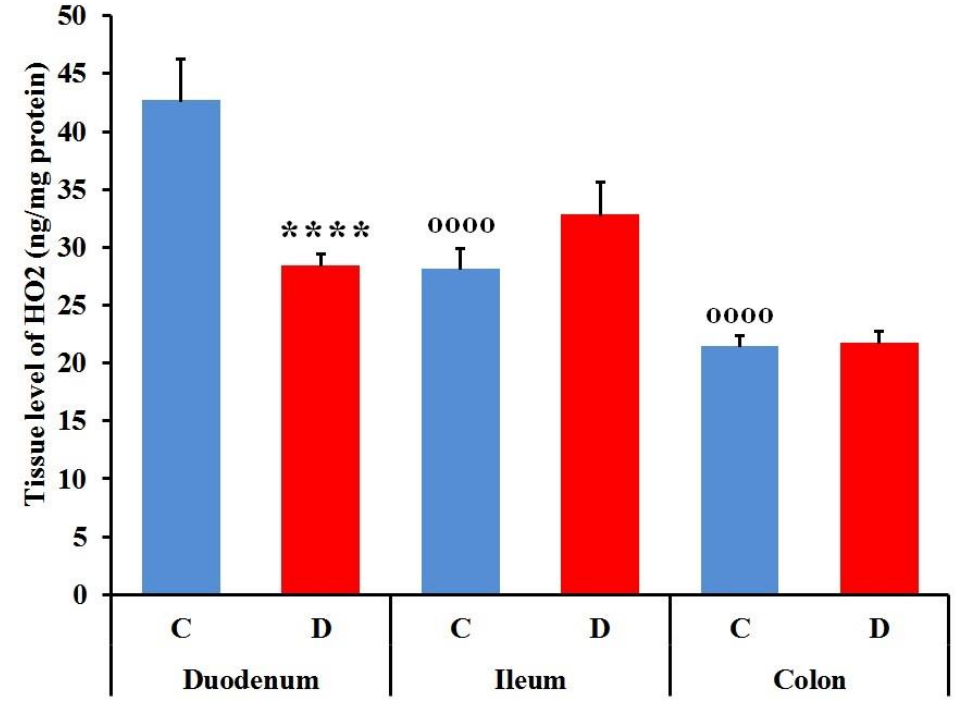


B

Figure 10. The tissue level of HO1 (A) and HO2 (B) in the different gut segments of control and diabetic rats. Data are expressed as mean ± SEM. ********P<0.0001 (controls vs. diabetics), ^{0000}P<0.0001 (between different gut segments of controls). C - controls; D - diabetics.

4.5 Evaluation of myenteric ganglia representing HO1-IR and nNOS-HO1-IR neurons

Nitrergic myenteric neurons were labelled by nNOS immunohistochemistry, whereas HO-representing neurons were labelled by HO1 and HO2 immunohistochemistry. The densities of HO1- and HO2-IR as well as HO1- and HO2-representing nitrergic neurons were evaluated per ganglia of duodenum, ileum and colon in control and diabetic group (Figure 11A,B).

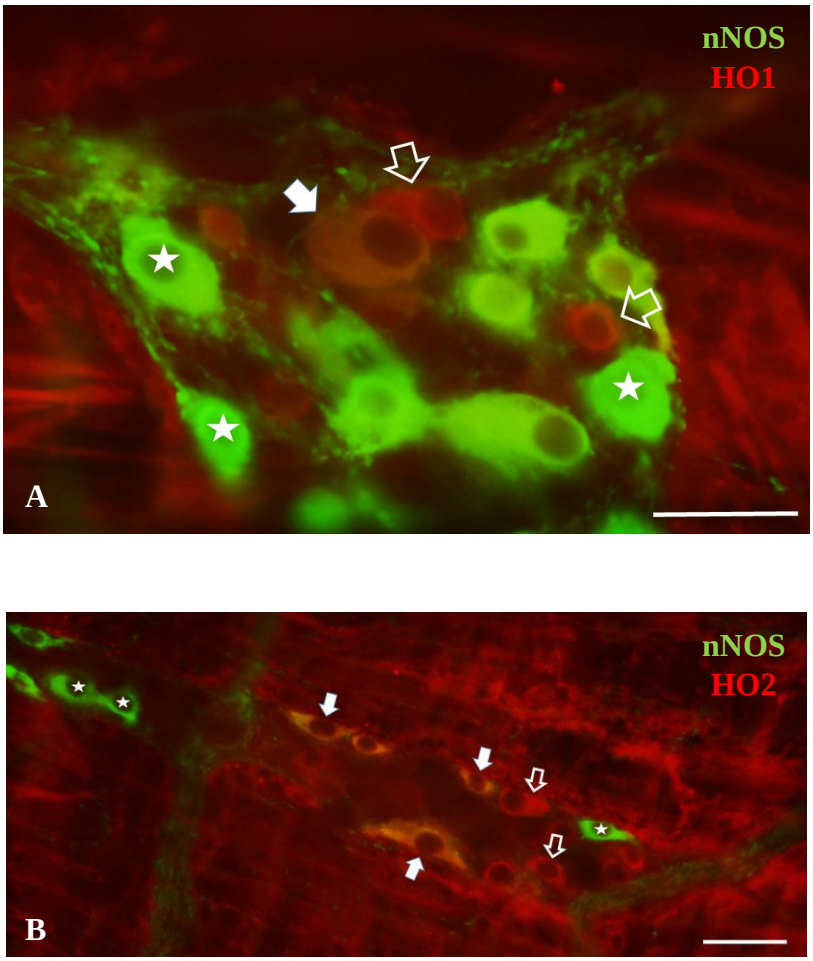
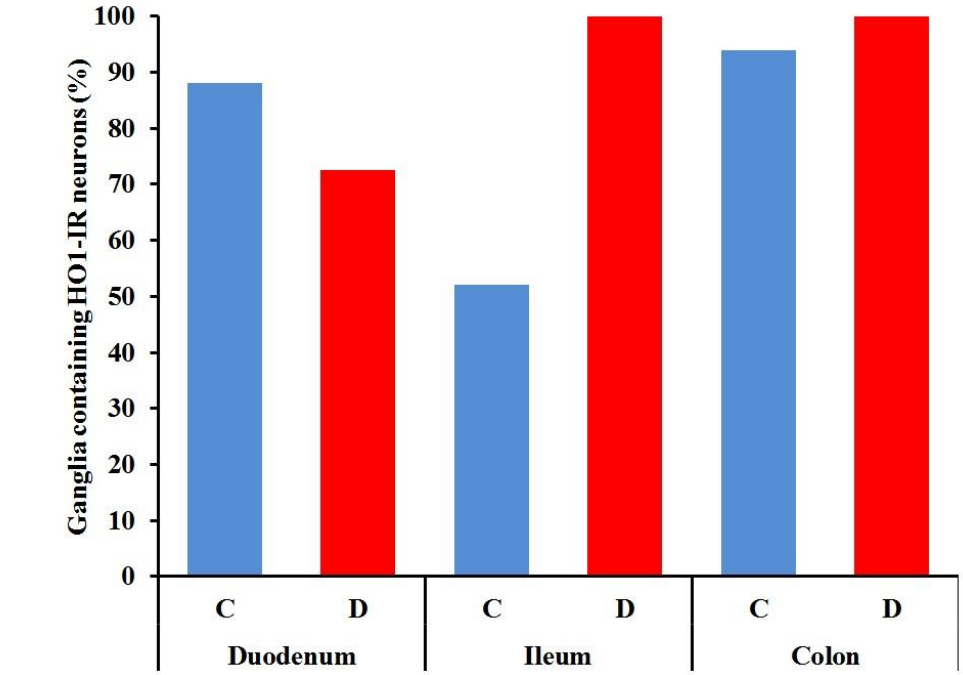


Figure 11. Representative fluorescent micrograph of whole-mount preparations of myenteric ganglia from a diabetic rat after nNOS-HO immunohistochemistry.
A) Stars indicate neurons that labelled for nNOS only, open arrows show neurons that labelled for HO1 only, and the solid arrow points to a myenteric neuron that double-labelled for both nNOS and HO1.
B) Stars indicate neurons that labelled for nNOS only, open arrows show neurons that labelled for HO2 only, and solid arrows point to duodenal myenteric neurons that double-labelled for both nNOS and HO2. Scale bars: 50 μm.

The myenteric ganglia containing HO1-IR and those ganglia with nNOS-HO1-IR neurons were varied from segments to segments in the control and diabetic group (Figure 12A, B). The highest percentage of HO1-IR neurons were observed in the colon (94%), followed by duodenum (88%), and ileum (52%) of control rats. In the diabetic group, the proportion of HO1-IR neurons was highly existing in the myenteric ganglia of colon and ileum (100%), whereas in the duodenum it was observed with 72.54%. Overall, the ganglia containing HO1-IR neurons in the diabetic ileum (100%) showed highest percentage differences as compared to the control ileum (52%).

The proportion of nNOS-HO1-IR neurons was high in the colon (78%) and duodenum (72%), but it was low in the ileum (16%) of controls. In the diabetic group, all the nitrergic neurons were stained as HO1-IR neurons (100%) in the colon, whereas ileum contained (61.76%) and duodenum (60.78%) double-stained neurons. Overall, HO1 containing nitrergic neurons in the myenteric ganglia of diabetic ileum showed highest percentage differences as compared to the control ileum.



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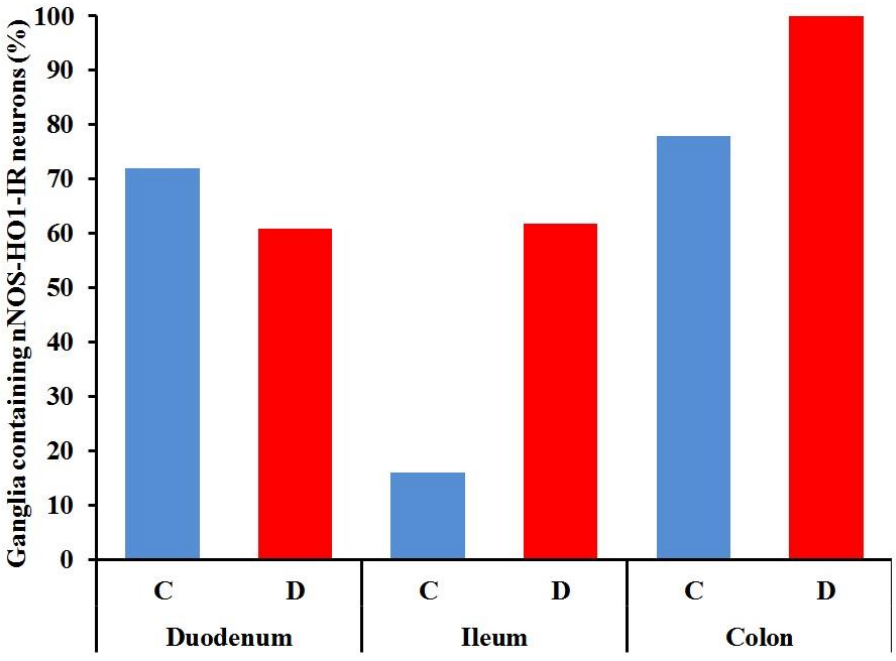


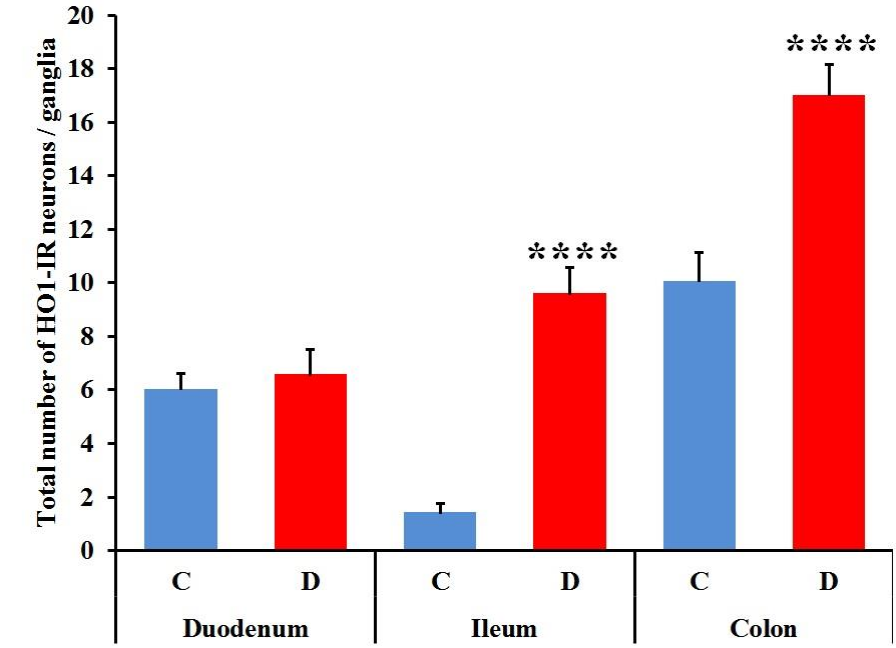
B

Figure 12. Percentage of myenteric ganglia containing HO1-IR (A) and nNOS-HO1-IR (B) neurons in the different intestinal segments of control and diabetic group. IR - Immunoreactive; C - controls; D - diabetics.

Total number of HO1-IR and HO1 representing nitrergic neurons

The presence of HO1-IR neurons varied distinctly in the different gut segments even in control animals. The number of HO1-IR cells was very low in the ileum (1.44±0.31), whereas the number of HO1-IR neurons were 4-fold higher in the duodenum (6.04±0.59) and 7-fold more in the myenteric ganglia of control colon (10.08±1.05). Except for the duodenum, the total number of HO1-IR neurons displayed a robust increase in the ileum ($P<0.0001$) and colon ($P<0.0001$) of diabetics as compared to the control group. The largest increase was observed in the ileum, whereas 7-fold more HO1-IR neurons were counted in the diabetics relative to controls (9.62±0.95 vs. 1.44±0.31) (Figure 13A).

Similar to the distribution of HO1-IR neurons, the number of nNOS-HO1-1R neurons was also the lowest in the ileum (0.20±0.06) and highest in the colon (2.22±0.27), whereas the number of double-stained neurons was slightly less in the duodenum (1.78±0.24) compared to the colon of controls. Moreover, dramatic increase was also observed by 7-fold in the ileum ($P<0.01$) of diabetics as compared to the controls (1.41±0.27 vs. 0.2±0.06 cells/ganglia). In the diabetic group, the number of nNOS-HO1-1R neurons were more than the double in the colon ($P<0.0001$), whereas in the duodenum it remained unchanged as compared to the control group. Since the number of total HO1-IR and nNOS-HO1-IR neurons showed similar alterations in the different gut segments of control and diabetic rats (Figure 13B).



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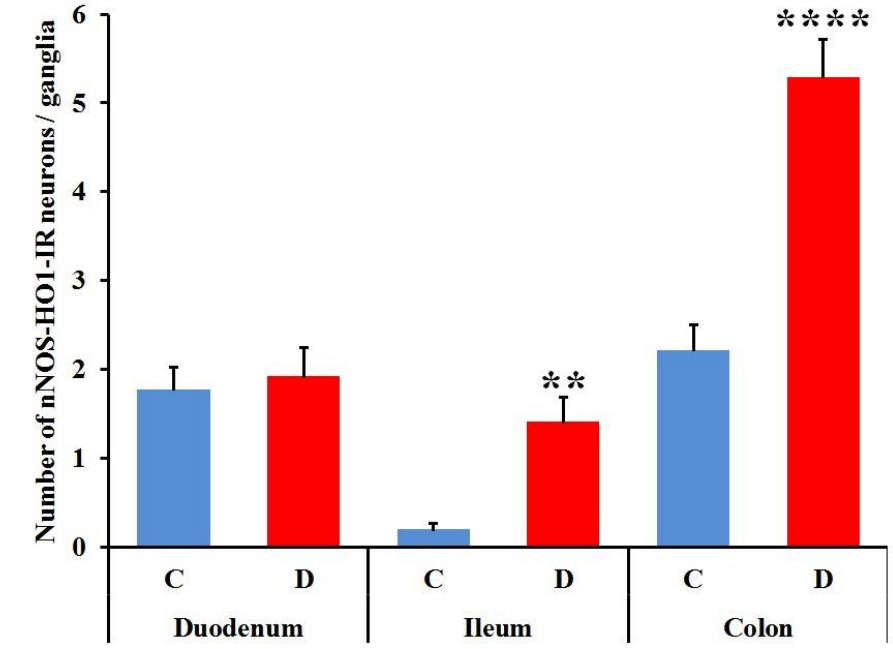


B

Figure 13. Quantitative evaluation of the total number of HO1-IR neurons (A), the number of nNOS-HO1-IR neurons (B) in the different gut segment of control and diabetic rats. Data are expressed as mean ± SEM. **P<0.01, ****P<0.0001 (controls vs. diabetics). IR - Immunoreactive; C - controls; D - diabetics.

The proportion of HO1-IR neurons co-localising with nNOS was observed with same frequency in the duodenum, ileum and colon of diabetics as compared to the control group. Overall, in the ileum only 18% of HO1-IR neurons are co-localised with nNOS, whereas this ratio was around 30% in the ganglia of duodenum and colon under both experimental conditions. (Figure 14A).

Although the total number of nNOS-IR neurons decreased in all intestinal segments (9±0.39 vs. 7.53±0.29 in the duodenum, $P<0.01$; 6.32±0.35 vs. 5.78±0.27 in the ileum; 9.5±0.48 vs. 7.38±0.42 in the colon, $P<0.001$), the proportion of nNOS-IR neurons co-localizing with HO1 exhibited a nearly 8-fold increase in the ileum and a 3-fold increase in the colon of diabetics. This ratio was barely 3% in the ileum and 23% in the colon of control rats, whereas it was 24% and 72% in the diabetics, respectively (Figure 14B).

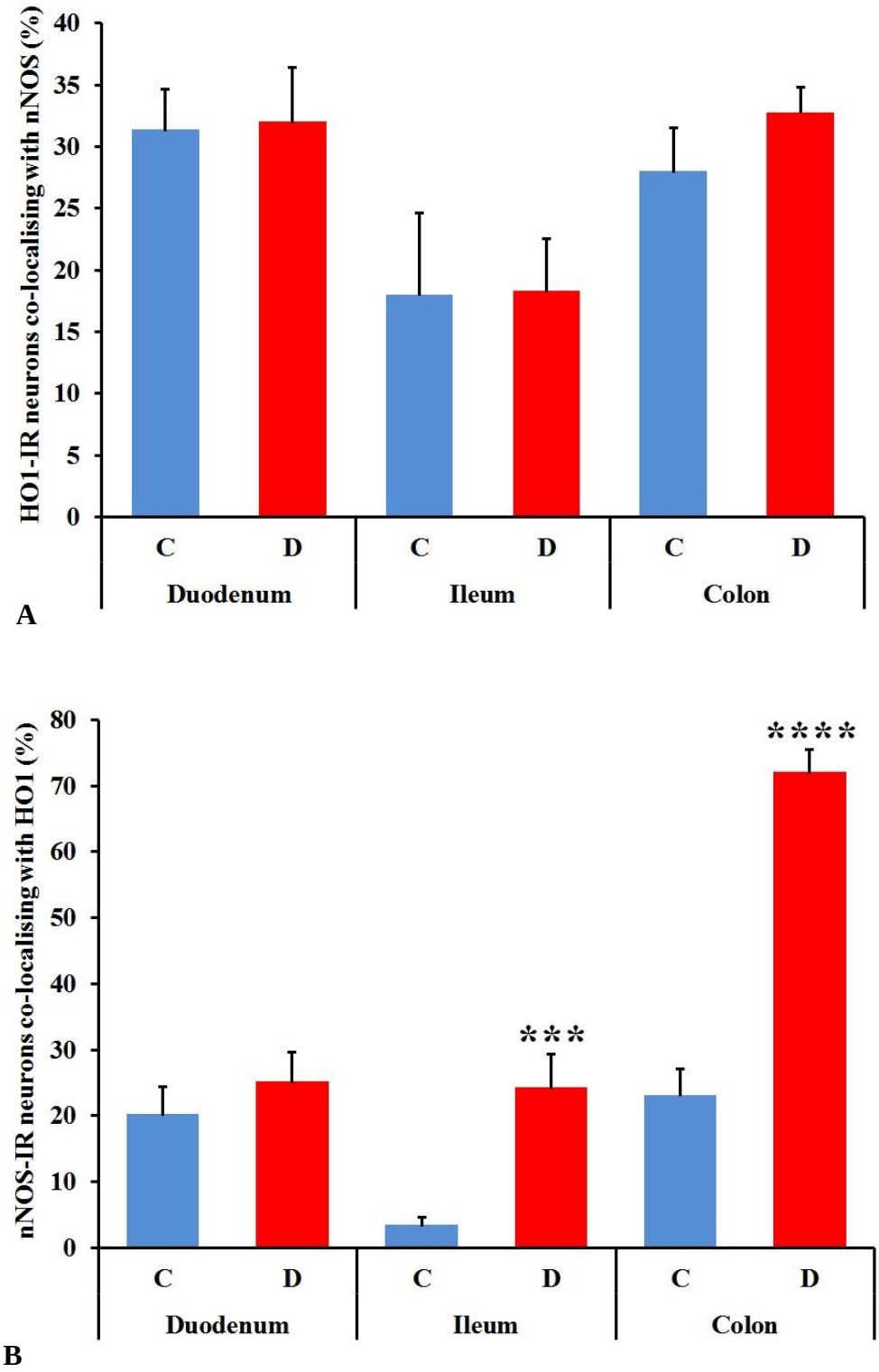
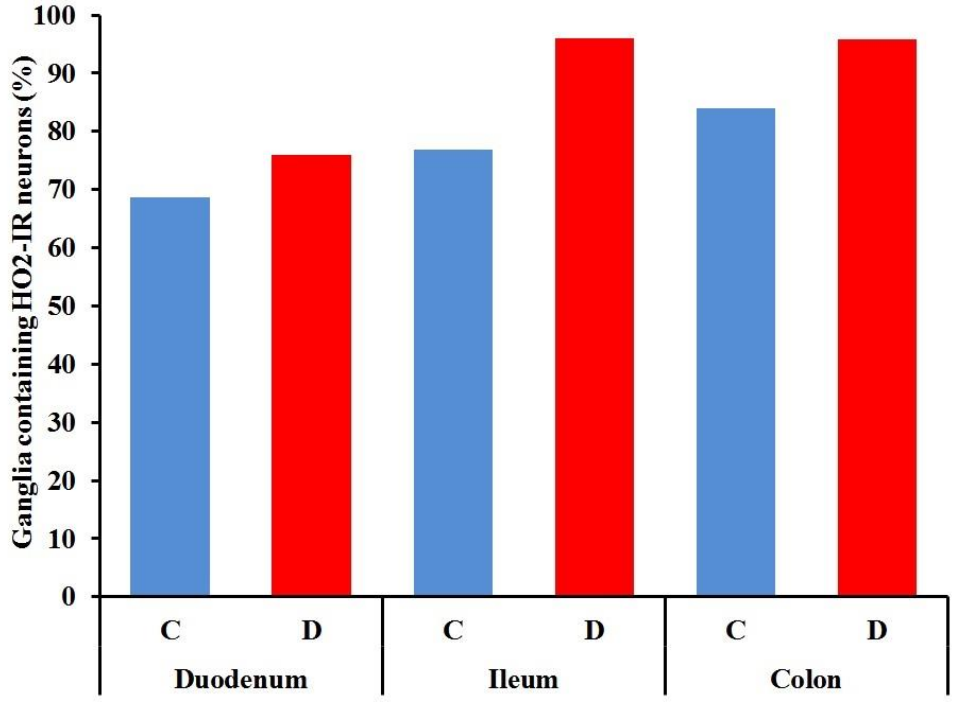


Figure 14. The proportion of HO1-IR neurons co-localizing with nNOS (A) and the proportion of nNOS-IR neurons co-localizing with HO1 (B) in the myenteric ganglia of the duodenum, ileum and colon of control and diabetic rats. Data are expressed as means ± SEM. ***P<0.001, ****P<0.0001 (controls vs. diabetics). IR - Immunoreactive; C - controls; D - diabetics.

4.6 Evaluation of myenteric ganglia representing HO2-IR and nNOS-HO2-IR neurons

The ganglia containing HO2-IR and nNOS-IR-HO2 myenteric neurons also varied in region-specific manner along the gut (Figure 15A). The proportion of HO2-IR myenteric neurons were increasing from proximal to distal part of control (68-84%) and diabetic group (76-95%). In the diabetic group, the proportion of HO2-IR myenteric neurons were dramatically increased and evenly present in the colon (95.92%) and ileum (95.74%), whereas in the duodenum it was observed with 76%. Moreover, the highest percentage differences were observed in the diabetic ileum as compared to the control ileum (Figure 15A).

The proportion of nNOS-HO2-IR-neurons were highly observed in the colon (80%), followed by duodenum (41.18%), and ileum (26.92%) in the myenteric ganglia of controls. In the diabetic group, the nitrergic neurons containing HO2-IR was 93.62% in the colon, whereas 81.63% in the ileum, and 60% in the duodenum. The highest percentage differences were observed in the diabetic ileum (81.63%) as compared to the control ileum (26.92%) (Figure 15B).



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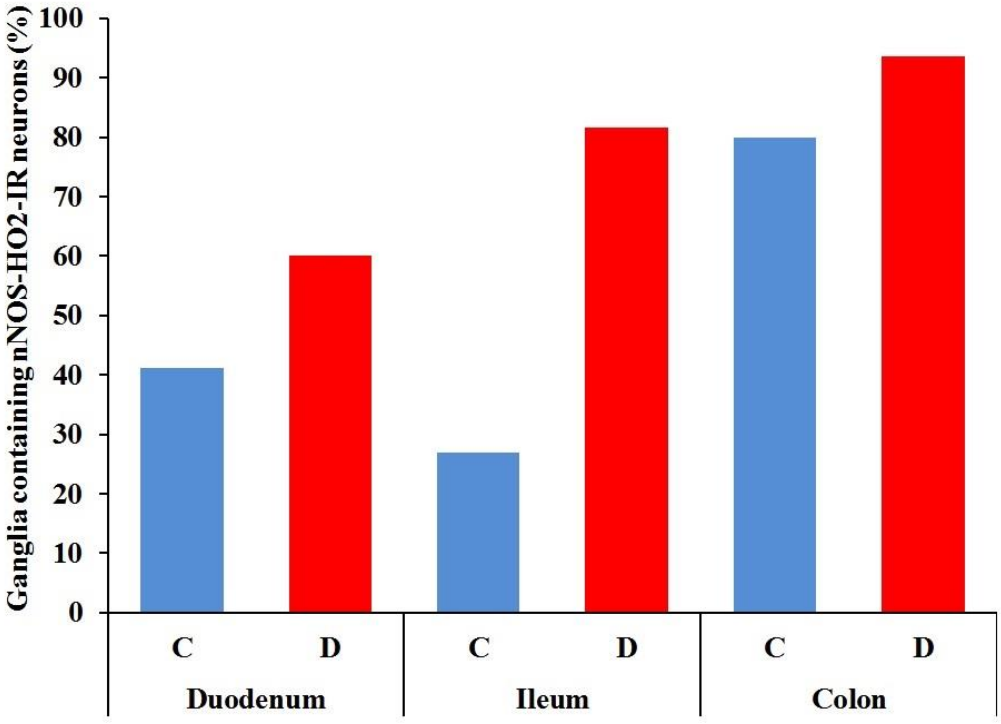


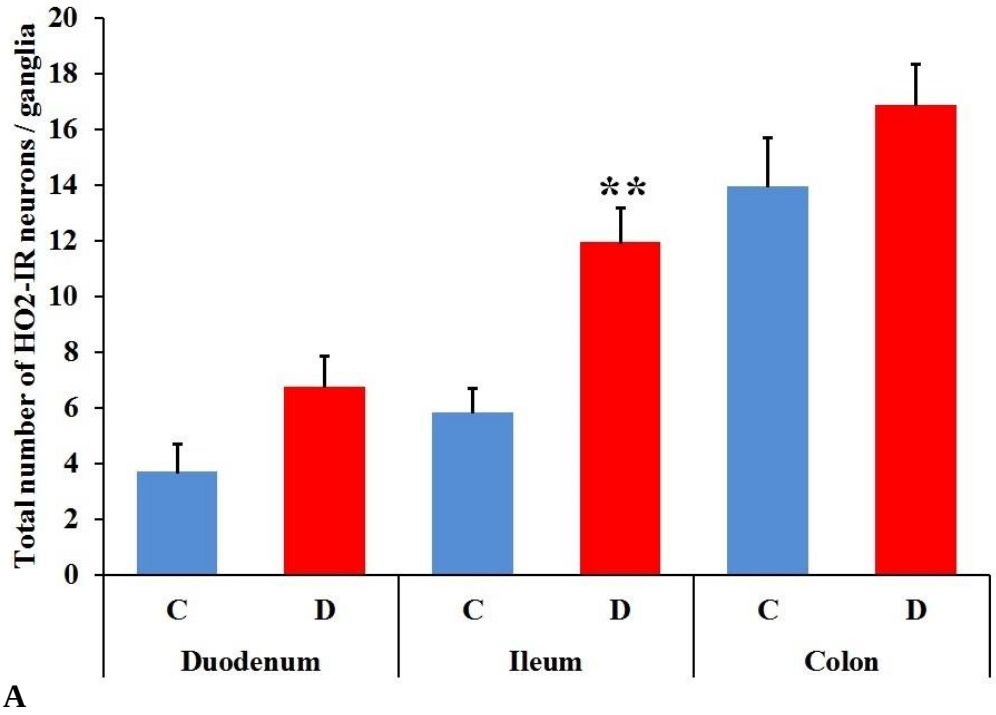
B

Figure 15. Percentage of myenteric ganglia containing HO2-IR (A) and nNOS-HO2-IR (B) neurons in the different intestinal segments of control and diabetic group. IR - Immunoreactive; C - controls; D - diabetics.

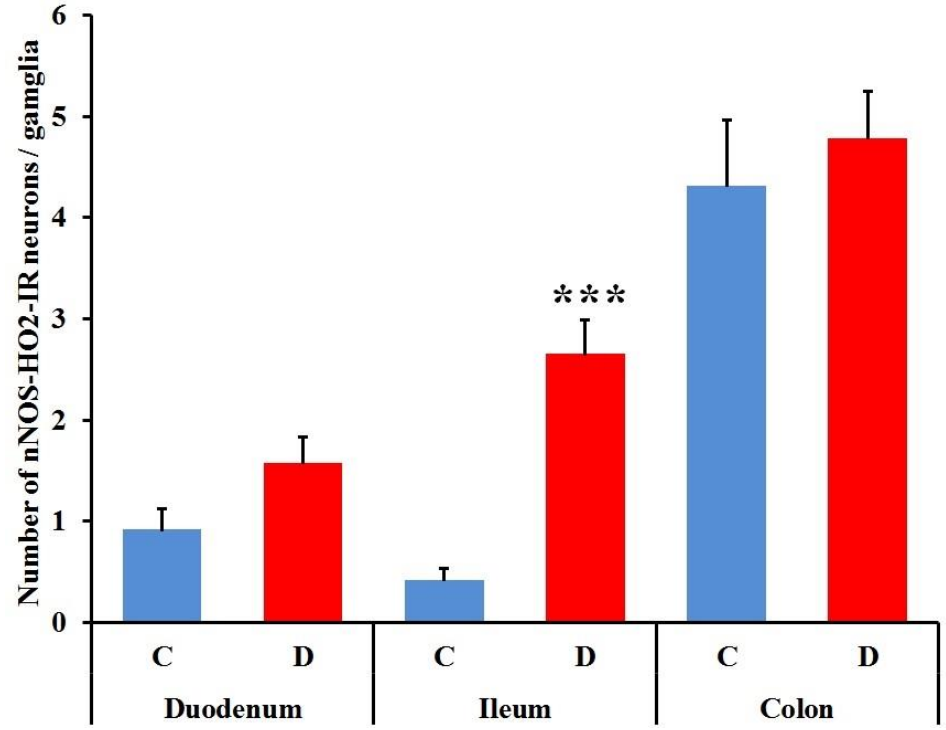
Proportion of HO2-IR- and HO2-representing nitrergic myenteric neurons

The distribution of HO2-IR neurons also displayed a region-dependent pattern in control rats. The number of HO2-IR neurons were highly present in the colon (16.89%) followed by ileum (5.85%), and duodenum (3.71%) of controls. In the diabetic rats, the total number of HO2-IR neurons were increased in all the intestinal segments; however, the increase was only significant in the ileum ($P<0.01$), whereas the HO2-IR neuronal number was doubled relative to controls (11.94±1.24 vs. 5.85±0.85 cells/ganglia) (Figure 16A).

Like the distribution of HO2-IR neurons, the number of nNOS-HO2-IR myenteric neurons also represented similar expression in controls where the majority of the co-localized neurons also occurred in the ganglia of colon (4.32±0.64), whereas an average of 0–1 cells were found in the ileal and duodenal ganglia. Moreover, the number of nNOS-HO2 co-localized neurons was also elevated more than 6-fold in the ileum of diabetics as compared to controls (2.65±0.34 vs. 0.42±0.11; $P<0.001$). The diabetic group of colon and duodenum were observed with slight increase but they were not significant as compared to the controls (Figure 16B).



A



B

Figure 16. Quantitative evaluation of the total number of HO2-IR neurons (A), the number of nNOS-HO2-IR neurons (B) in the myenteric ganglia of the duodenum, ileum and colon of control and diabetic group. Data are expressed as means ± SEM. **P<0.01, ***P<0.001 (controls vs. diabetics). IR - Immunoreactive; C - controls; D - diabetics.

The proportion of HO2-IR neurons co-localised with nNOS showed highest percentage in the colon (39%) and duodenum (28%), whereas the lowest percentage was in the ileum (6.32%) of controls. In the diabetic group, the percentage of HO2-IR neurons co-localised with nNOS was increased by 4-fold in the ileum ($P<0.001$), whereas the duodenum and colon did not show any significant differences as compared to the controls (Figure 17A). Hence, the number of total HO2-IR and also that of the nNOS-HO2-IR neurons did not alter significantly in the duodenum and colon of control and diabetic group, therefore the proportion of HO2-IR neurons co-localizing with nNOS remained unchanged in these intestinal regions.

Although the total number of nNOS-IR neurons were decreased in all intestinal regions (10.39 ± 0.51 vs. 7.96 ± 0.44 in the duodenum, $P<0.01$; 7.33 ± 0.42 vs. 6.23 ± 0.22 in the ileum, $P<0.05$; 10.48 ± 0.69 vs. 8.08 ± 0.69 in the colon, $P<0.01$) (Figure 17B). However, the proportion of nNOS-IR neurons co-localizing with HO2 was dramatically increased in all gut segments of the diabetic group. The largest, nearly 7-fold increase in this ratio was observed in the ileum (40% vs. 6%), but significant increases were also shown in the duodenum (22% vs. 9%) and the colon (68% vs. 44%) of diabetics as compared to controls (Figure 17C).

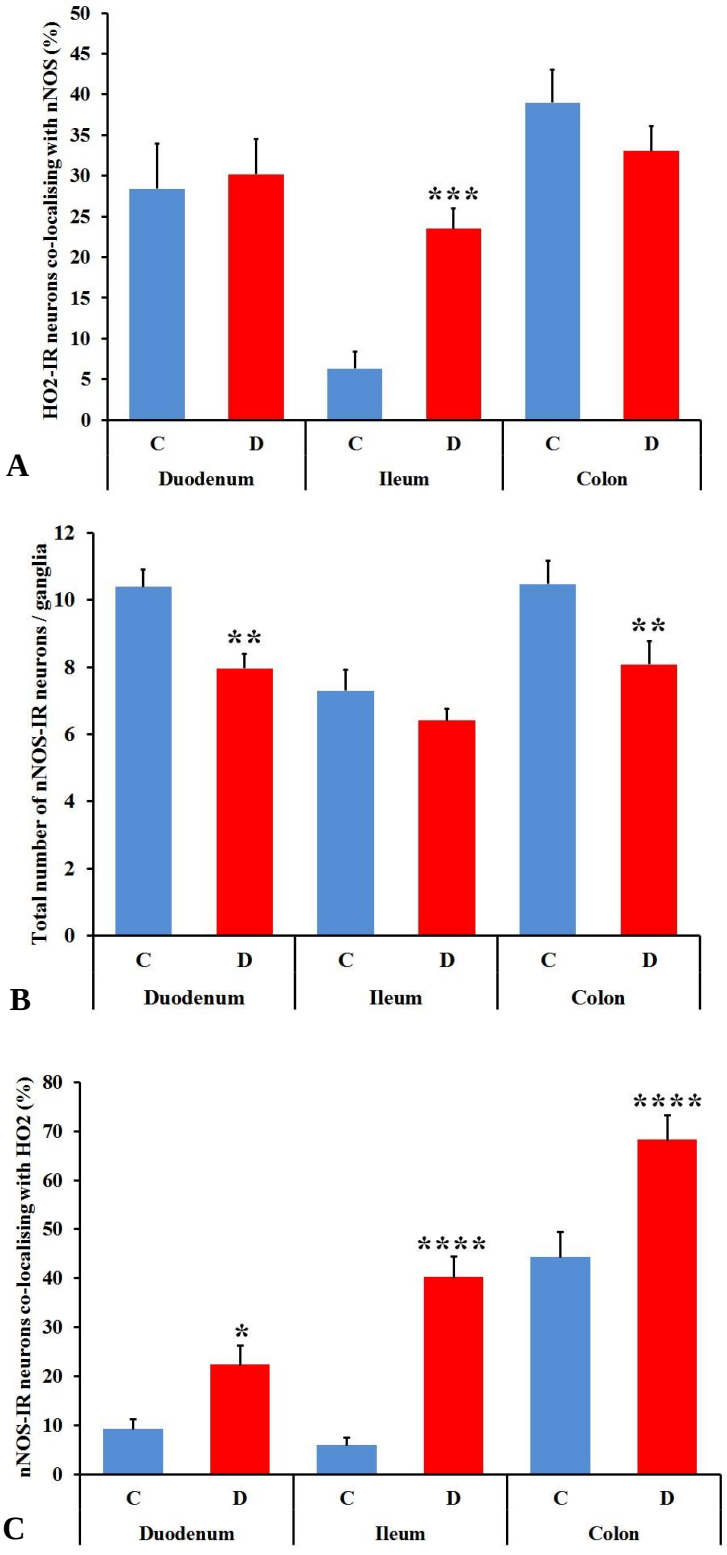


Figure 17. The proportion of HO2-IR neurons co-localizing with nNOS (A), the total number of nNOS-IR neurons per ganglia (B) and the proportion of nNOS-IR neurons co-localizing with HO2 (C) in the myenteric ganglia of the duodenum, ileum and colon of control and diabetic group. Data are expressed as means ± SEM. *****P<0.05, ******P<0.01, *******P<0.001 ********P<0.0001 (controls vs. diabetics). IR - Immunoreactive; C - controls; D - diabetics.

4.7 Evaluation of submucous ganglia representing nNOS-IR, HO1-IR and HO2-IR neurons

To determine the proportion of nNOS-IR neurons in the total submucous neuronal number, nNOS and HuC/D double-labelling fluorescent immunohistochemistry was applied, as shown in the representative micrograph (Figure 18). To visualize and quantify the occurrence of HO1-IR submucous neurons, HO1 immunohistochemistry was used combined with peripherin as pan-neuronal marker (Figure 19A). The occurrence of HO2-IR submucous neurons was also calculated as the proportion of peripherin-stained total submucous neuronal number (Figure 19B).

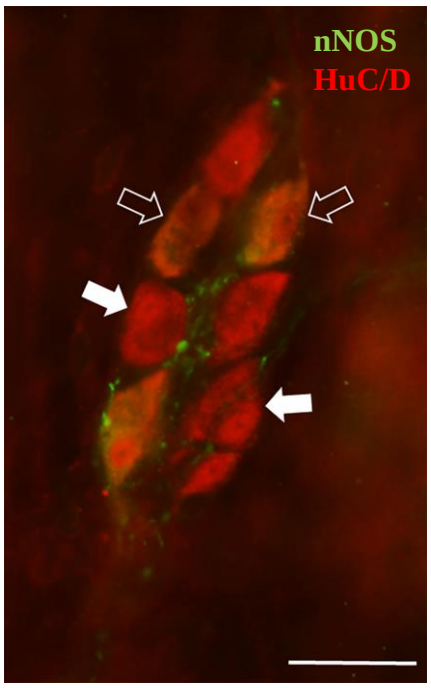


Figure 18. Representative fluorescent micrograph of a whole-mount preparation of submucous ganglia from the colon of a diabetic rat after nNOS-HuC/D double-labelling immunohistochemistry. Solid arrows indicate neurons labelled for HuC/D only, open arrows show neurons double-labelled for both nNOS and HuC/D; Scale bar: 50 μm.

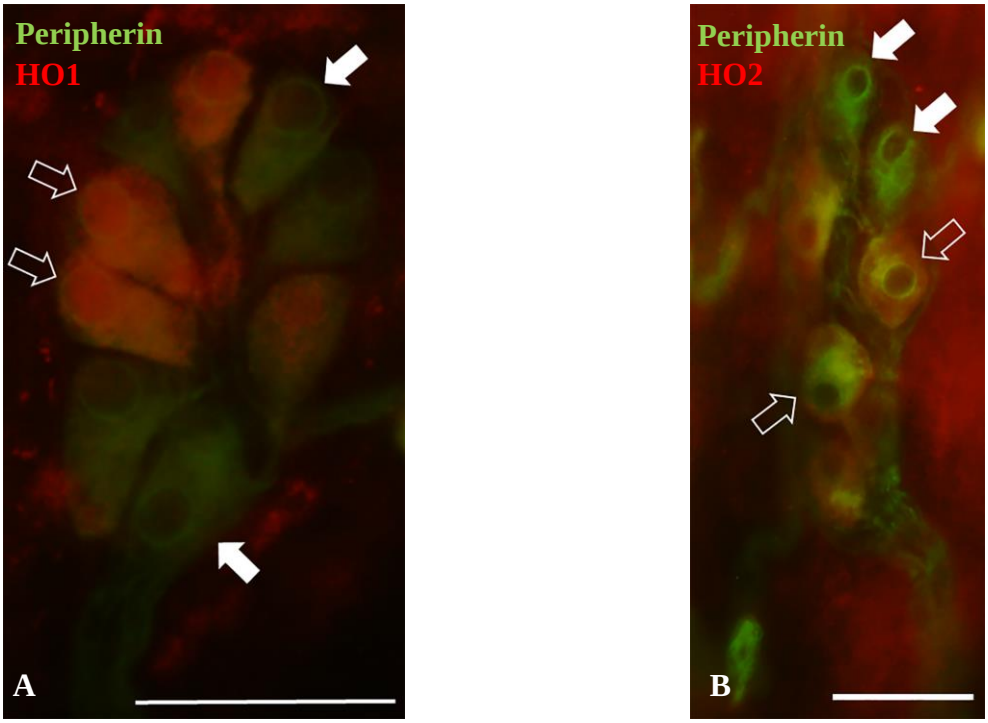


Figure 19. Representative fluorescent micrograph of a whole-mount preparation of submucous ganglia from the colon of a control rat after double-labelling immunohistochemistry.
A) Solid arrows indicate neurons labelled for peripherin only, open arrows show neurons double-labelled for both HO1 and peripherin.
B) Solid arrows indicate neurons labelled for peripherin only, open arrows show neurons double-labelled for both HO2 and peripherin; Scale bars: 50 μm.

Proportion of nNOS-IR submucous neurons

In the total number of neurons, no changes were found between the different gut segments within the control group or among the three experimental groups in the duodenal, ileal or colonic intestinal regions (Figure 20). The proportion of nitrergic neurons in the total neuronal number did not show any differences between the three investigated gut segments of controls (Figure 21A). STZ treatment resulted in an increase in the proportion of nitrergic neurons in the distal intestinal segments (in the ileum and colon) but not in the duodenum. The proportion of the nitrergic subpopulation exhibited a 2-fold increase in the ileum (12% vs. 6% in controls), whereas a 3-fold increase was observed in the colon of diabetics (22% vs. 8% in controls; Figure 21B). The insulin treatment had no effect on the proportion of duodenal and colonic nitrergic submucous neurons which, however, was found to increase significantly in the ileal segment as compared to the diabetic rats.

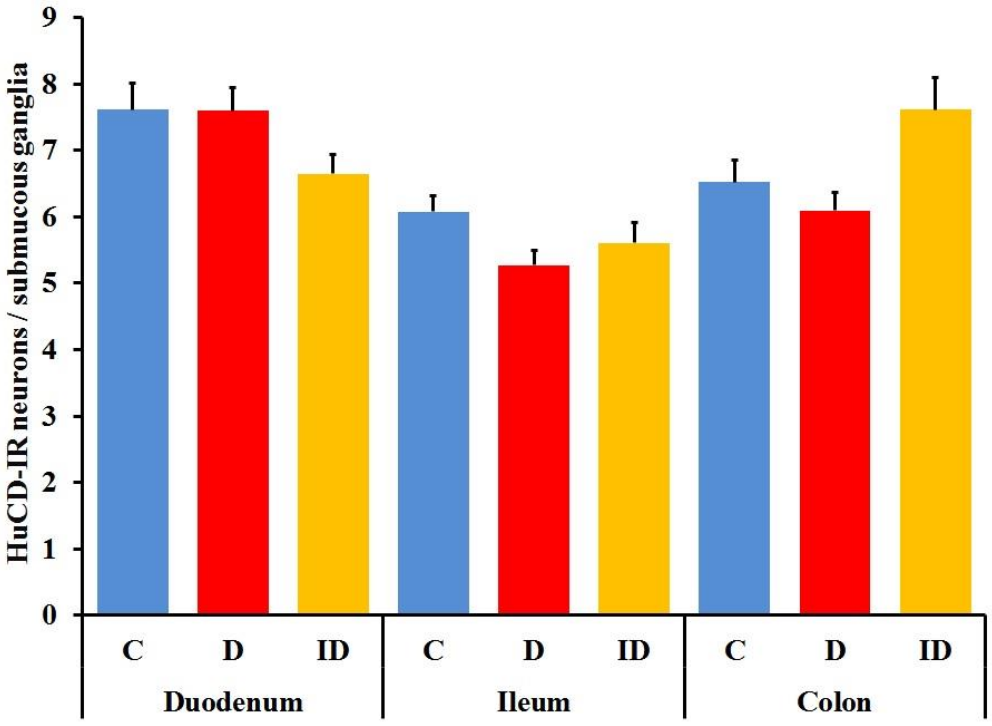


Figure 20. Total neuronal number in the submucous ganglia of duodenum, ileum and colon in the different experimental groups. Data are expressed as means ± SEM. C - controls; D - diabetics; ID - insulin-treated diabetics.

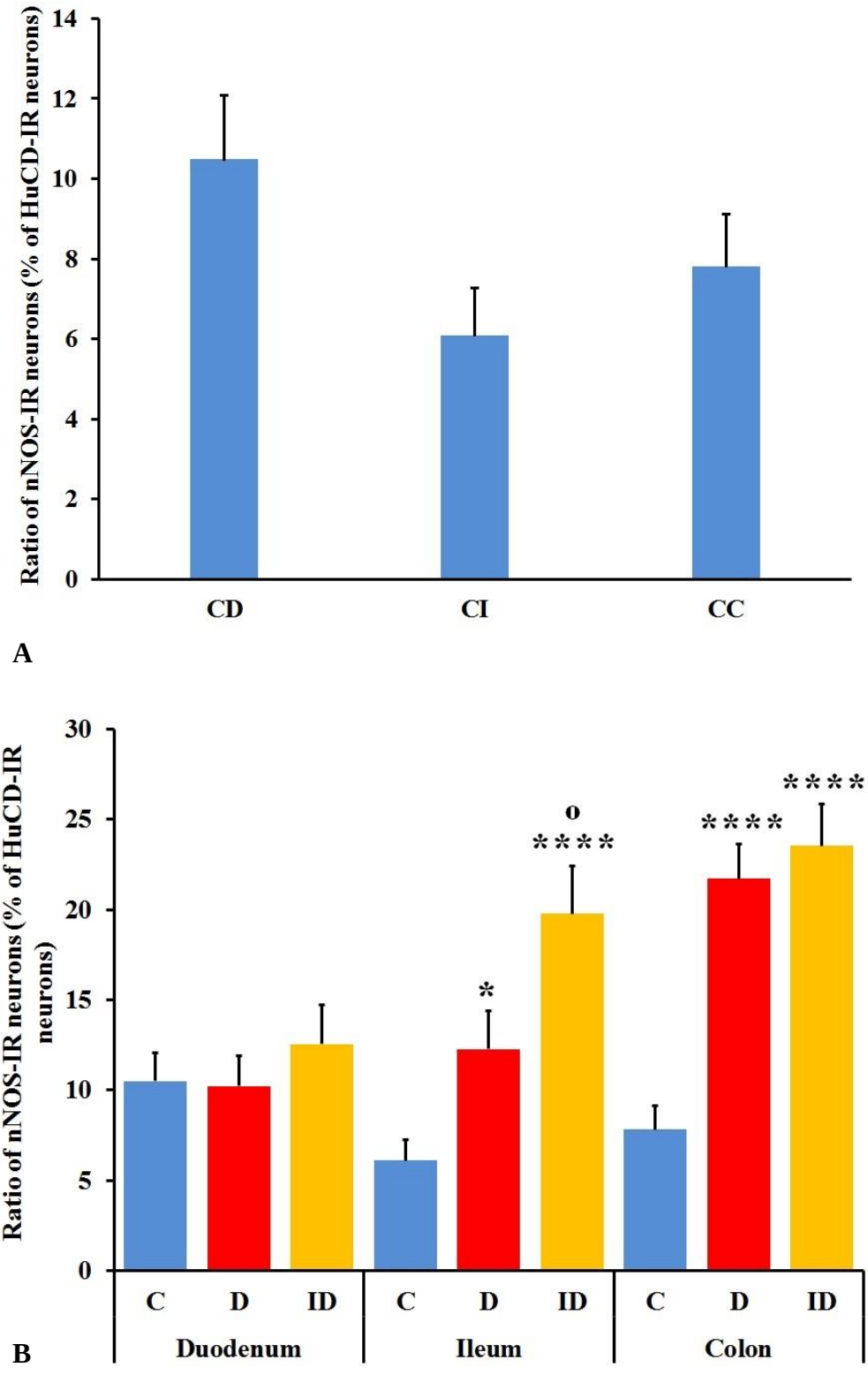


Figure 21. Proportion of nNOS-IR neurons related to the total number of submucous neurons in the duodenum, ileum and colon. **A**: Only in controls; **B**: in control, diabetic and insulin-treated diabetic rats. Data are expressed as mean ± SEM; *****P<0.05 and ********P<0.0001 vs. controls, ºP<0.05 vs. diabetics. IR - Immunoreactive; CD - control duodenum; CI - control ileum; CC - control colon; C - controls; D - diabetics; ID - insulin-treated diabetics.

Proportion of HO1-IR submucous neurons

The distribution of HO1-IR submucous neurons as compared to total neuronal number demonstrated region-dependent differences in the three examined segments even in the controls. The proportion of duodenal and ileal HO1-IR submucous neurons was only 4% and 0%, respectively, but the occurrence of HO1-IR neurons was extremely robust in the colon (51%; $P<0.0001$; Figure 22A). In the diabetics, results similar to the controls were obtained along the intestinal tract, with HO1-IR neurons seldom occurring in the small intestine and present in abundance in the large intestine (Figure 22B). In the duodenal segment no change was induced by the insulin treatment in the presence of HO1-IR neurons, whereas in the ileum quantitative analysis of the HO1-IR submucous subpopulation revealed a significant increase in the proportion of these neurons in the insulin-treated diabetic group (6%) as compared to the control group (0%). In the colonic submucous ganglia, we found a significant decrease in the insulin-treated diabetics (14%) as compared to the control (51%) or the diabetic group (38%) (Figure 22B).

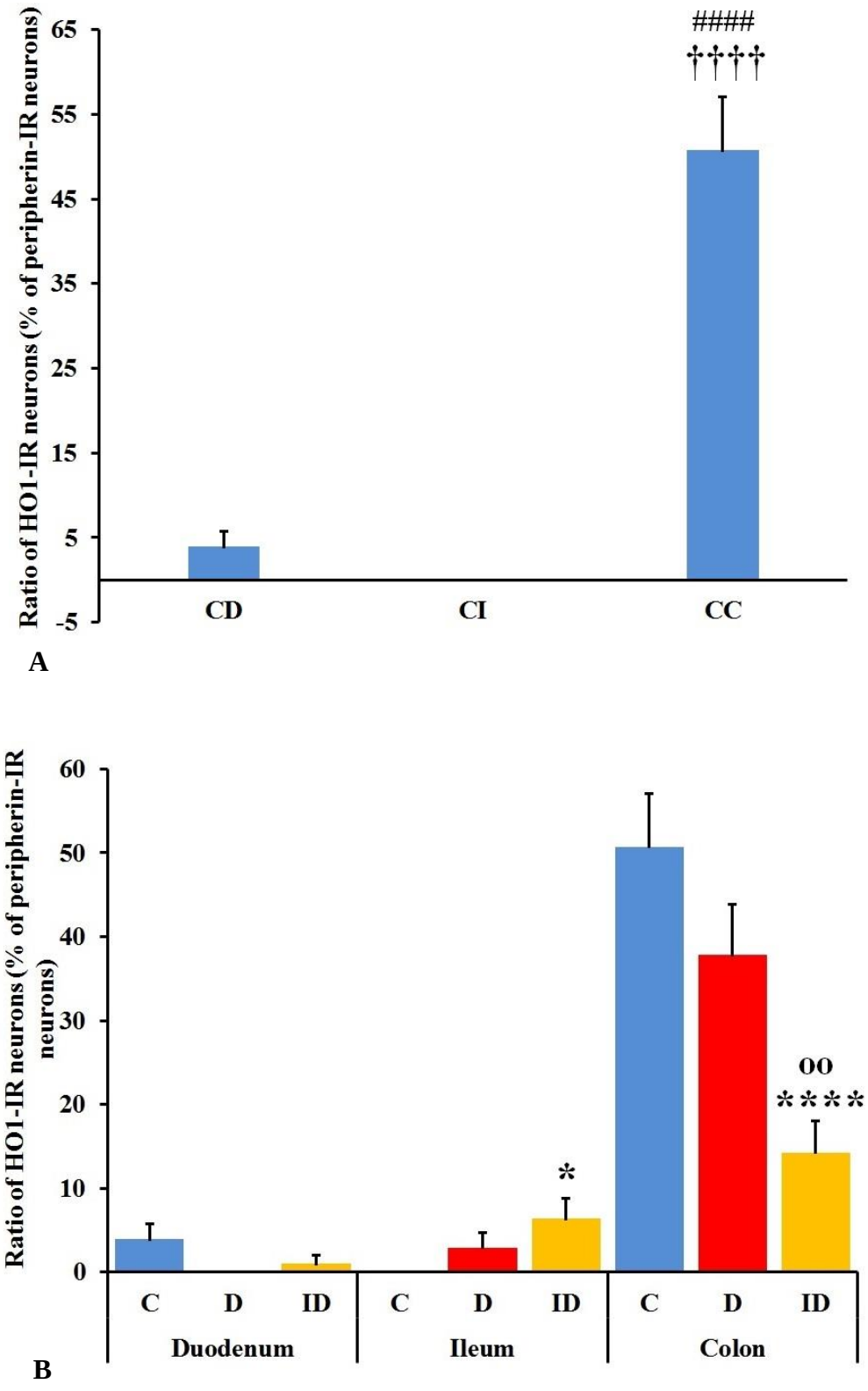


Figure 22. Proportion of HO1-IR neurons related to the total number of submucous neurons in the duodenum, ileum and colon. **A**: Only in controls; **B**: In control, diabetic and insulin-treated diabetic rats. Data are expressed as mean ± SEM; ††††P<0.0001 vs. control duodenum (CD); ####P<0.0001 vs. control ileum (CI), control colon (CC); *****P<0.05 and ********P<0.0001 vs. controls, and **ºº**P<0.01 vs. diabetics. IR - Immunoreactive; C - controls; D - diabetics; ID - insulin-treated diabetics.

Proportion of HO2-IR submucous neurons

In the controls, the HO2-IR neurons demonstrated a distribution similar to that of the HO1-IR submucous neurons. In the segments of small intestine, a low level of HO2-IR neurons was detected (4% in the duodenum, 1% in the ileum), but in the colon this proportion was significantly higher (38%; $P<0.0001$; Figure 23A).

In the diabetics, results similar to the controls were obtained along the intestine; the marked differences in the ratio of HO2-IR neurons between the different gut segments remained unchanged (Figure 23B).

Immediate insulin replacement had no effect on the abundance of HO2-IR neurons in the duodenum. Although the ileal segment showed the lowest proportion of HO2-IR neurons compared to the other two control regions, a significant increase in their numbers was found in the ileum after insulin replacement (17%) compared to the control (1%) and the diabetic rats (6%). The proportion of colonic HO2-IR neurons showed a pattern similar to that of the HO1-IR neurons in the same segment. The 10-week insulin treatment reduced the proportion of HO2-IR neurons (28%) below the control level, and this decrease was significant as compared to the diabetic group (52%; Figure 23B).

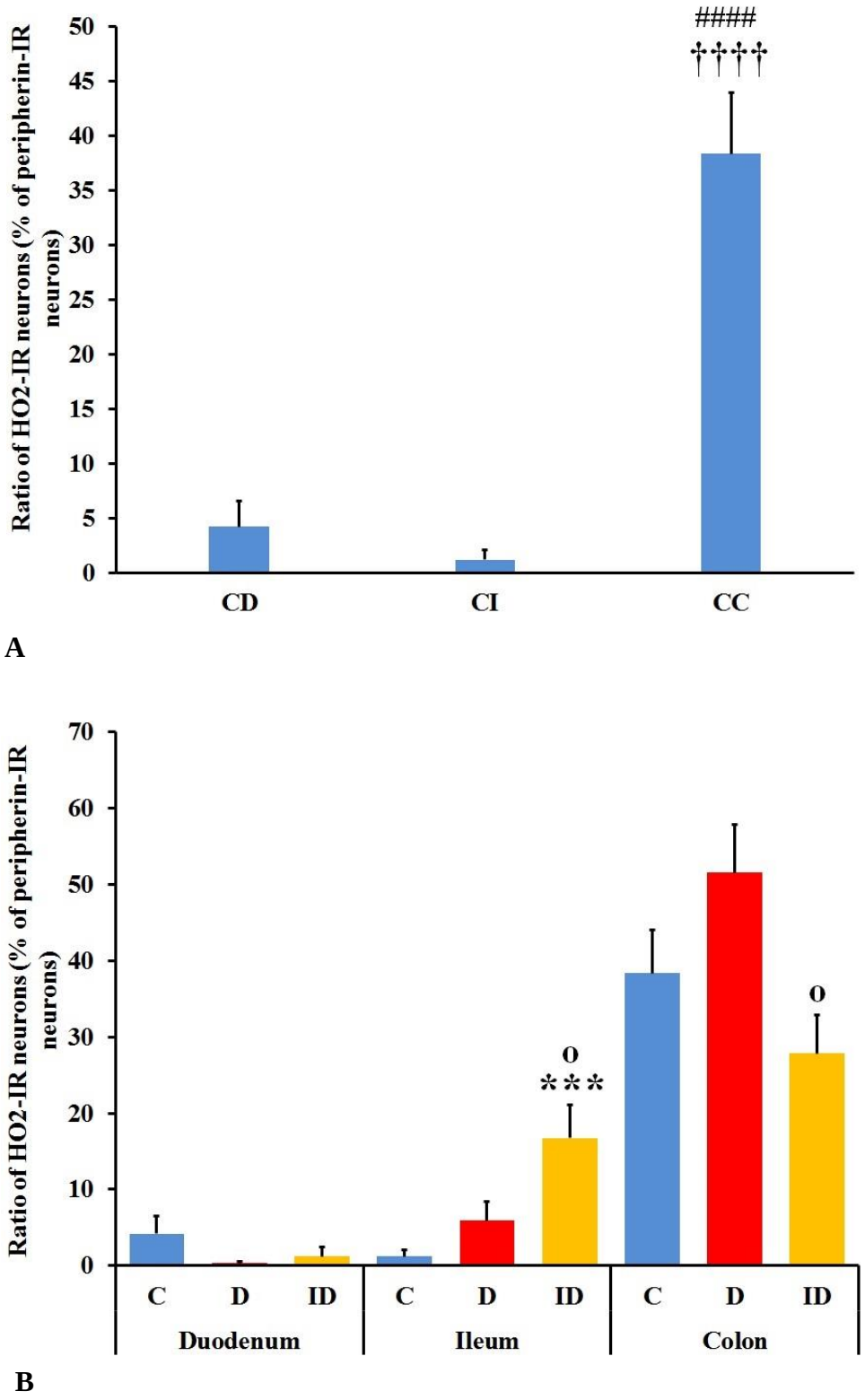


Figure 23. Proportion of HO2-IR neurons related to the total number of submucous neurons in the duodenum, ileum and colon. **A**: Only in controls; **B**: In control, diabetic and insulin-treated diabetic rats. Data are expressed as means ± SEM; ††††P<0.0001 vs. control duodenum (CD), ####P<0.0001 vs. control ileum (CI), control colon (CC); ***P<0.001 vs. controls, and ºP<0.05 vs. diabetics; IR - Immunoreactive; C - controls; D - diabetics.; ID - insulin-treated diabetics.

4.8 Tissue levels of pro-inflammatory cytokines in the smooth muscles and the myenteric plexus

TNFα

In the tissue homogenates including the smooth muscle layers of the intestinal wall and the myenteric plexus, the expression pattern of TNFα, IL1β, and IL6 were strictly region dependent in the control rats. Under control condition, TNFα concentration was highly detected in the ileum (269.1±24.48 pg/mg of protein), whereas it was slightly less in the duodenum (213.1±23.68 pg/mg of protein) and very low in the colon (153.5±17.23 pg/mg of protein).

In the diabetic group, the tissue levels of TNFα was significantly increased only in the duodenum (356.1± 36.93 pg/mg of protein), whereas in the ileum (231.1±26.84 pg/mg of protein) and colon, its levels (170.9±12.69 pg/mg of protein) were unaffected by diabetes compared to controls (Figure 24A).

The immediate insulin treatment induced massive changes in the TNFα concentration only in the duodenal segment. It showed 3-fold increase in the duodenum (635.1±41.91 pg/mg of protein) than that of the controls (213.1±23.68 pg/mg of protein) and 2-fold increase compared to diabetic (356.1±36.93 pg/mg of protein) group. (Figure 24A).

IL1β

The highest concentration of IL1β was detected in the ileum (0.48±0.07 pg/mg of protein) of controls, whereas the duodenum (0.30±0.04 pg/mg of protein) and colon (0.32±0.07 pg/mg of protein) were maintained the same concentrations.

In the diabetic group, the duodenum was the only segment where the tissue levels of IL1β was increased significantly (0.75±0.14 pg/mg of protein), whereas in the colon (0.41±0.08 pg/mg of protein) it was slightly elevated without any significance and in the ileum (0.44±0.05 pg/mg of protein) IL1β levels was not affected by diabetes compared to control group (Figure 24B).

The immediate insulin treatment intensely increased the concentration of IL1β in the duodenum (0.73±0.08 pg/mg of protein; $P<0.05$), whereas it did not cause any changes in the ileum (0.45±0.05 pg/mg of protein) and colon (0.39± 0.03 pg/mg of protein) compared to controls (Figure 24B).

IL6

The tissue level of IL6 was highly detected in the ileum (106.30±7.72 pg/mg of protein) of controls followed by duodenum (83.41±13.50 pg/mg of protein) and colon (55.29±4.18 pg/mg of protein).

In the diabetic group, the concentration of IL6 was decreased in the ileal segment (75.82±11.98 pg/mg of protein; $P<0.05$), whereas it was unaltered in the duodenum (99.35±13.88 pg/mg of protein) and in the colon (53.72±9.32 pg/mg of protein) compared to controls (Figure 24C).

The immediate insulin treatment elevated slightly the levels of IL6 in the duodenum (124.30±13.08 pg/mg of protein) and decreased significantly in the ileum (70.83±4.72 pg/mg of protein), whereas it did not show any changes in the colon (58.90± 2.61 pg/mg of protein) compared to controls (Figure 24C).

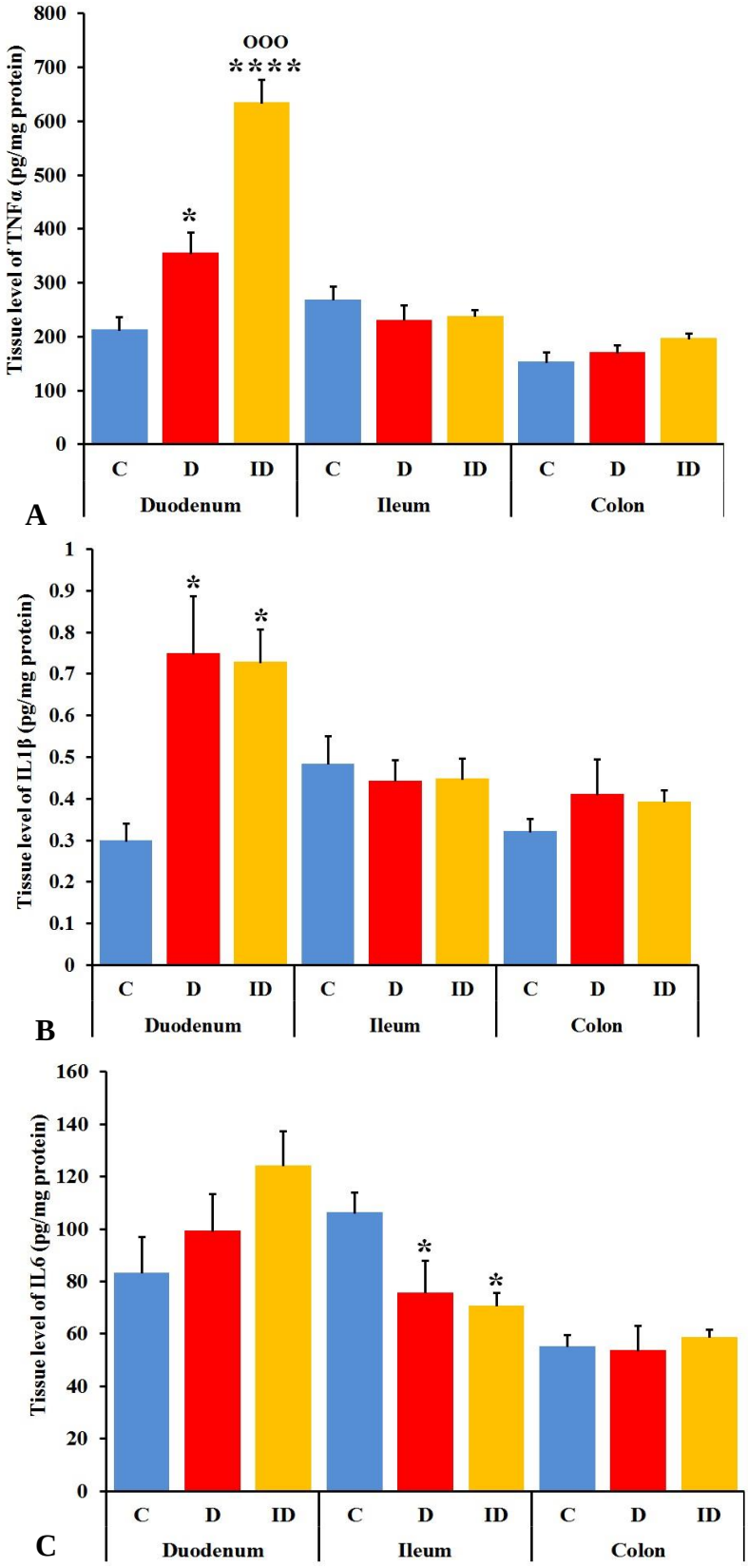


Figure 24. Tissue levels of pro-inflammatory cytokines in the myenteric plexus, circular and-longitudinal smooth muscles. **TNFα (A), IL1β (B), IL6 (C).** Data are expressed as means ± SEM. *P<0.05, ****P<0.0001 (compared to controls); °°°P<0.001 (diabetics vs. insulin-treated diabetics). C - controls; D - diabetics; ID - insulin-treated diabetics.

4.9 Tissue levels of pro-inflammatory cytokines in the mucosa-submucous plexus

TNFα

In the tissue homogenates of mucosa-submucosa with the submucous plexus, the expression of TNFα were strictly region dependent in the control rats. The concentration of TNFα was more than double as well as maintained at same level in the ileum (15.37±0.4705 pg/mg protein) and colon (15.36±0.795) compared to duodenum (6.098±0.645 pg/mg protein) of controls. In the diabetics, the tissue level of TNFα was significantly decreased in the ileum (6.097±1.077 pg/mg protein; ***P<0.001) and colon (6.183±0.6496 pg/mg protein; ***P<0.001), whereas in the duodenum (7.843±1.063 pg/mg protein) it was slightly increased without any significance compared to control group (Figure 25).

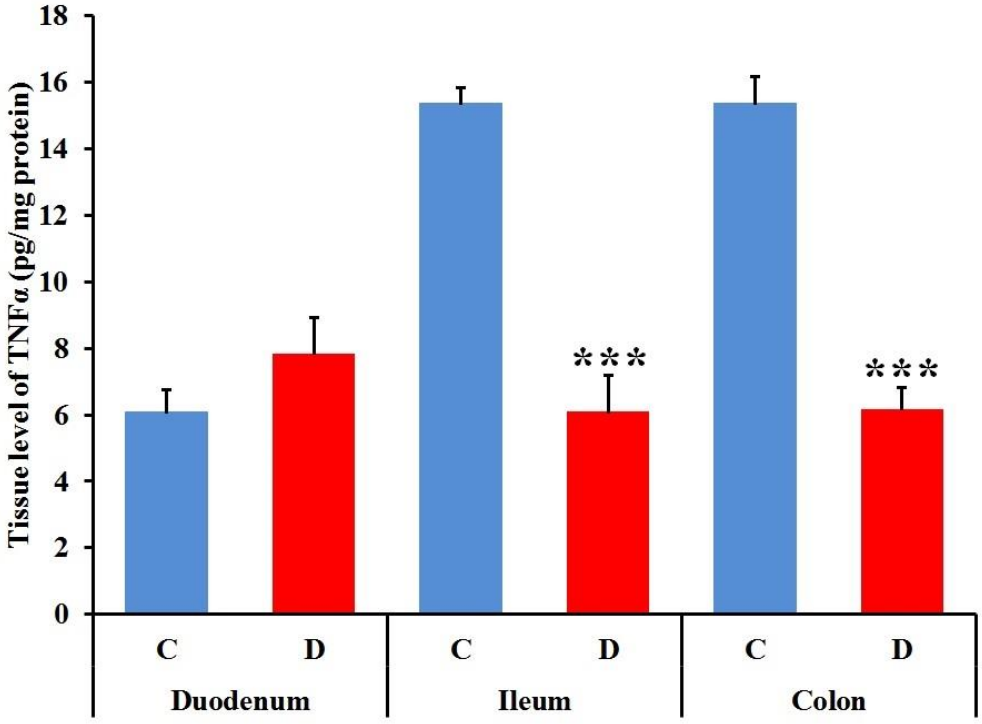


Figure 25. Tissue level of TNFα in the mucosa-submucous plexus. Data are expressed as means ± SEM. ***P<0.001 (compared to controls); C - controls; D - diabetics.

IL1β and IL6

The tissue levels of IL1β and IL6 were undetectable in the mucosa-submucosa of control and diabetic group.

4.10 Intracellular distribution of TNFα and IL6 in the myenteric ganglia and capillary endothelium

TNFα and IL6 were labelled by post-embedding 18 nm gold particles and the density of these gold particles were evaluated per area in the myenteric ganglia and capillary endothelium of duodenum, ileum, and colon in control, diabetic and insulin-treated diabetic group.

Distribution of TNFα- and IL6-labelling gold particles in the myenteric ganglia of different experimental groups

The density of TNFα- and IL6-labelling gold particles was strictly region-dependent, with increasing from proximal duodenum to distal colon of controls. The TNFα-labelling gold particles were distributed in small group on the membrane-bound organelles like mitochondria, Golgi-apparatus, and endoplasmic reticulum in the myenteric ganglia of different intestinal segments (Figure 26).

The number of TNFα gold particles was significantly increased in the myenteric ganglia of colon (1.37±0.17/μm^2) and ileum (1.09± 0.22/μm^2) compared to duodenum (0.47± 0.06/μm^2) of control group (Figure 27A). The number of gold particles revealed a 2-fold increase in the duodenal (0.83±0.09/μm^2), decreased significantly in the colonic (0.71±0.07/μm^2) and did not show any significant changes in the ileal (1.24±0.16/μm^2) myenteric ganglia of diabetics compared to control group (Figure 27B).

The immediate insulin treatment caused significant increase in the number of gold particles in the duodenum (0.91±0.13/μm^2) and decrease in the colon (0.95±0.11/μm^2) compared to control group. In the ileum (0.59±0.08/μm^2) of insulin-treated diabetics, the number of gold particles was decreased significantly compared to control (1.09±0.22/μm^2) and diabetic group (1.24±0.16/μm^2) (Figure 27B).

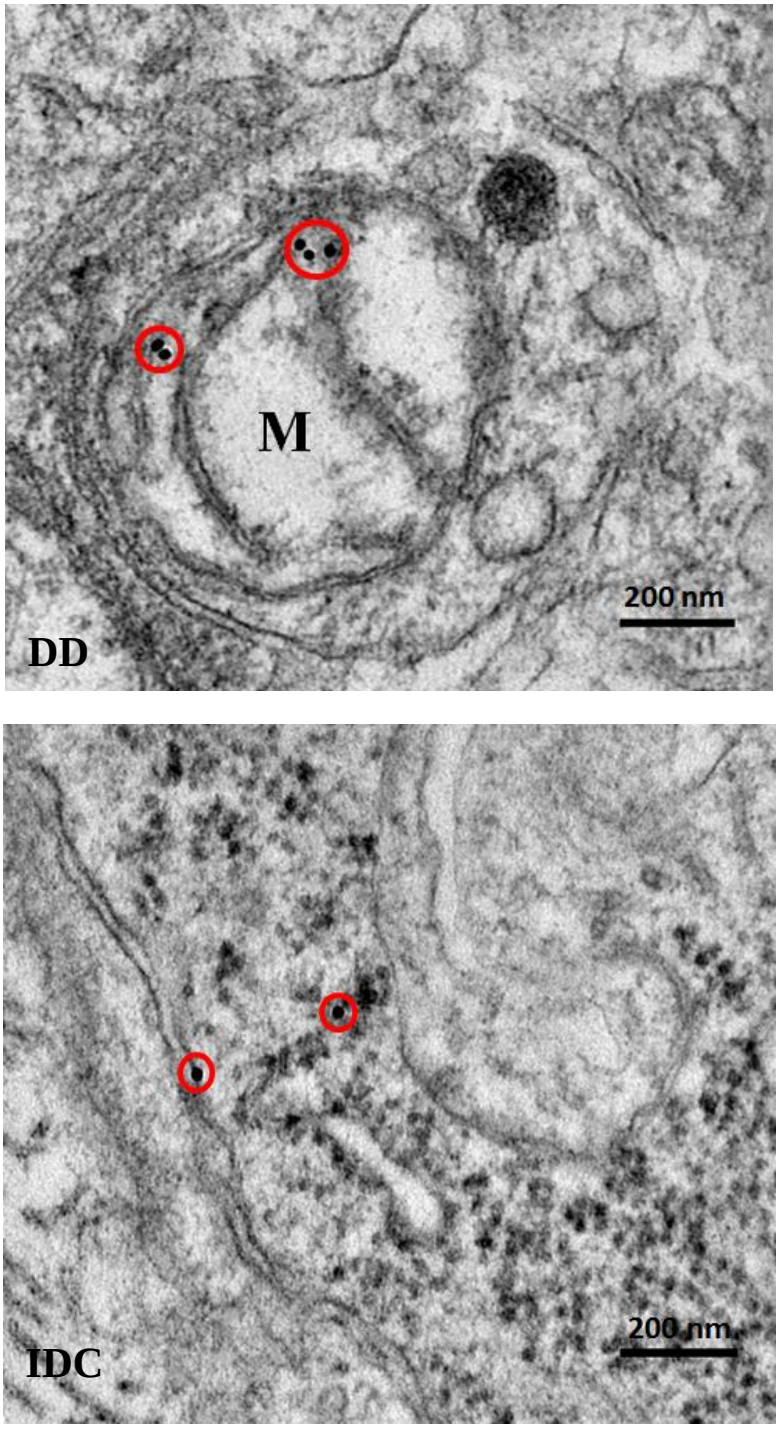


Figure 26. Representative electron micrographs of ultrathin sections of myenteric ganglia from diabetic duodenum (DD) and insulin-treated diabetic colon (IDC); Mitochondria - M. The TNFα was labelled by 18 nm gold particles; shown in circle.

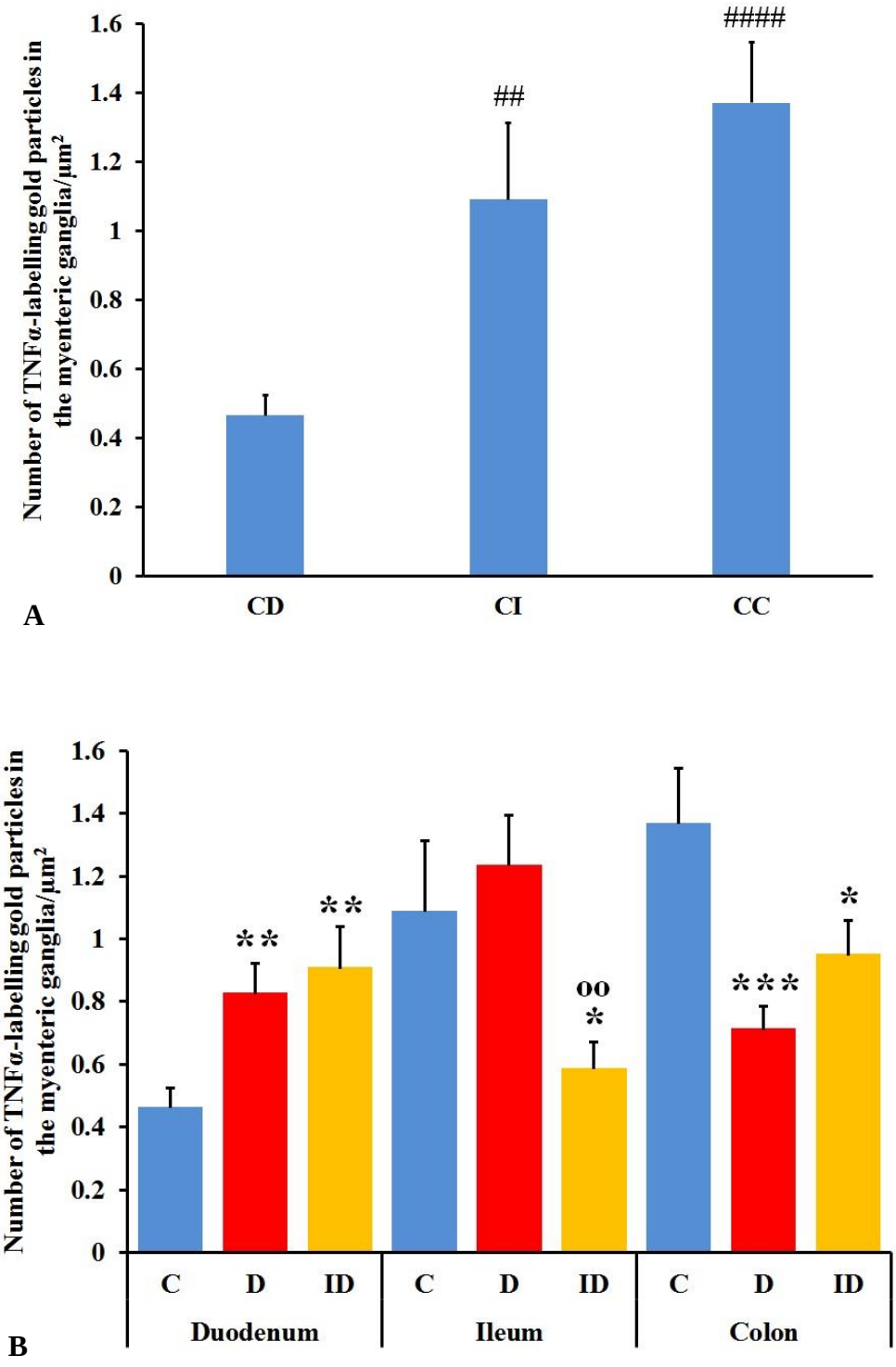


Figure 27. Number of TNFα-labelling gold particles in the myenteric ganglia of different gut segments in controls (A) and under different experimental condition (B). Data are expressed as means ± SEM. $^{\#\#}P<0.01$,$^{\#\#\#\#}P<0.0001$ (compared to duodenum); CD - control duodenum; CI - control ileum; CC - control colon. $^{*}P<0.05$,$^{**}P<0.01$,$^{***}P<0.001$ (compared to controls); $^{oo}P<0.01$ (diabetics vs. insulin-treated diabetics). C - controls; D - diabetics; ID - insulin-treated diabetics.

The density of IL6-labelling gold particles was also distributed on the mitochondria, Golgi apparatus, endoplasmic reticulum, and ribosomes in the myenteric ganglia from different intestinal segments (Figure 28). The number of IL6 gold particles was increasing from proximal to distal throughout the length of the gastrointestinal tract, whereas significant differences were observed in the myenteric ganglia of colon ($0.23\pm0.04/\mu m^2$) compared to duodenum ($0.12\pm0.03/\mu m^2$) and ileum ($0.14\pm0.03/\mu m^2$) of control group (Figure 29A).

In the diabetic group, the number of gold particles was elevated in all the gut segments, however, it did not show any significant differences compared to controls. The number of gold particles was robustly increased in the duodenal ($0.43\pm0.08/\mu m^2$; $P<0.01$) and slightly decreased in the colonic myenteric ganglia ($0.13\pm 0.03/\mu m^2$) of insulin-treated diabetic group. While in the ileum ($0.22\pm0.04/\mu m^2$) the number of gold particles was elevated in the insulin-treated diabetics but without any significant differences compared to the control group.

The density of gold particles was increased 2-fold ($0.43\pm0.08/\mu m^2$; $P<0.01$) in the duodenum and decreased significantly ($0.13\pm 0.03/\mu m^2$; $P<0.05$) in the colon of insulin-treated diabetic compared to diabetic group (Figure 29B).

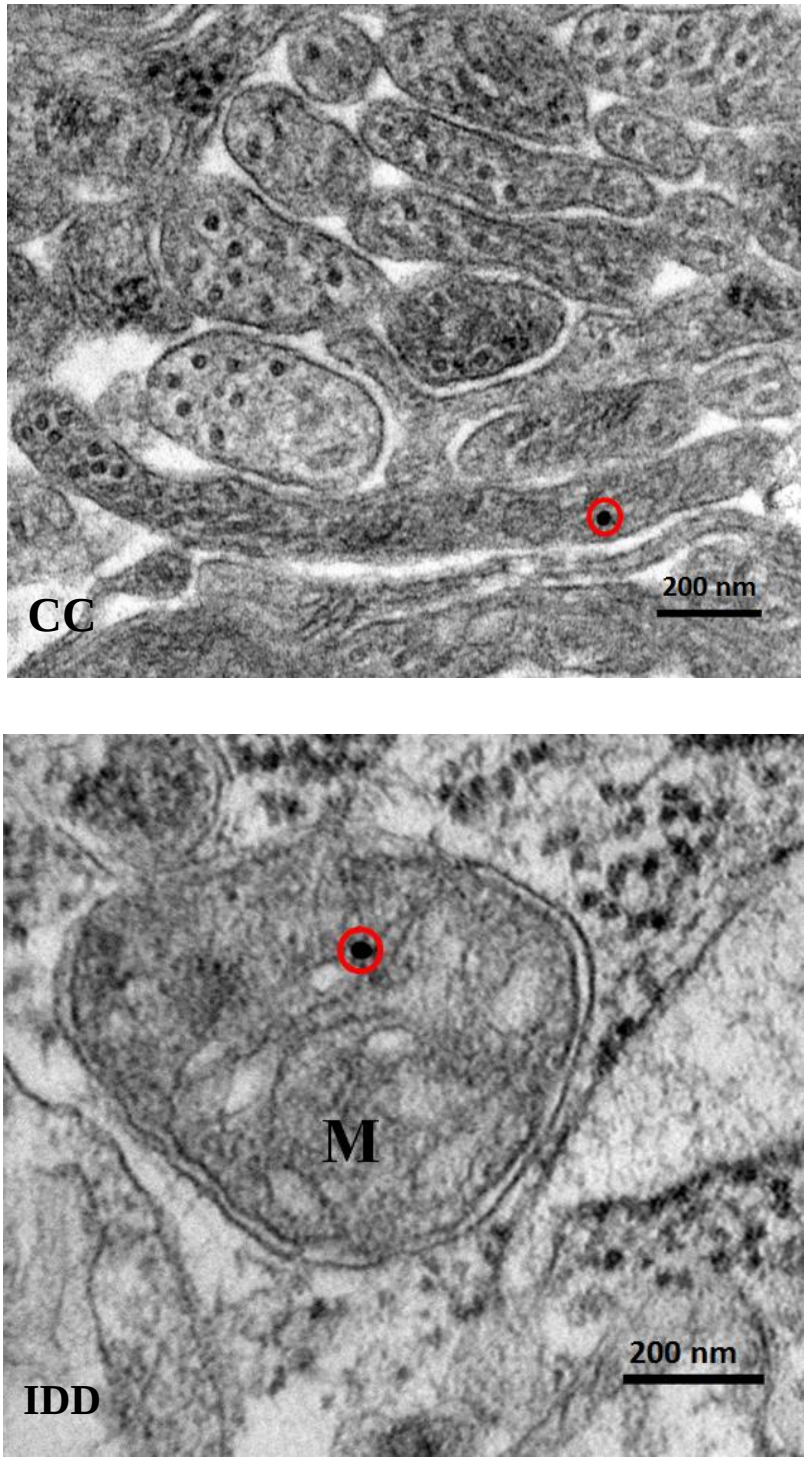


Figure 28. Representative electron micrographs of ultrathin sections of myenteric ganglia from control colon (CC) and insulin-treated diabetic duodenum (IDD); Mitochondria - M. The IL6 was labelled by 18 nm gold particles; shown in circle.

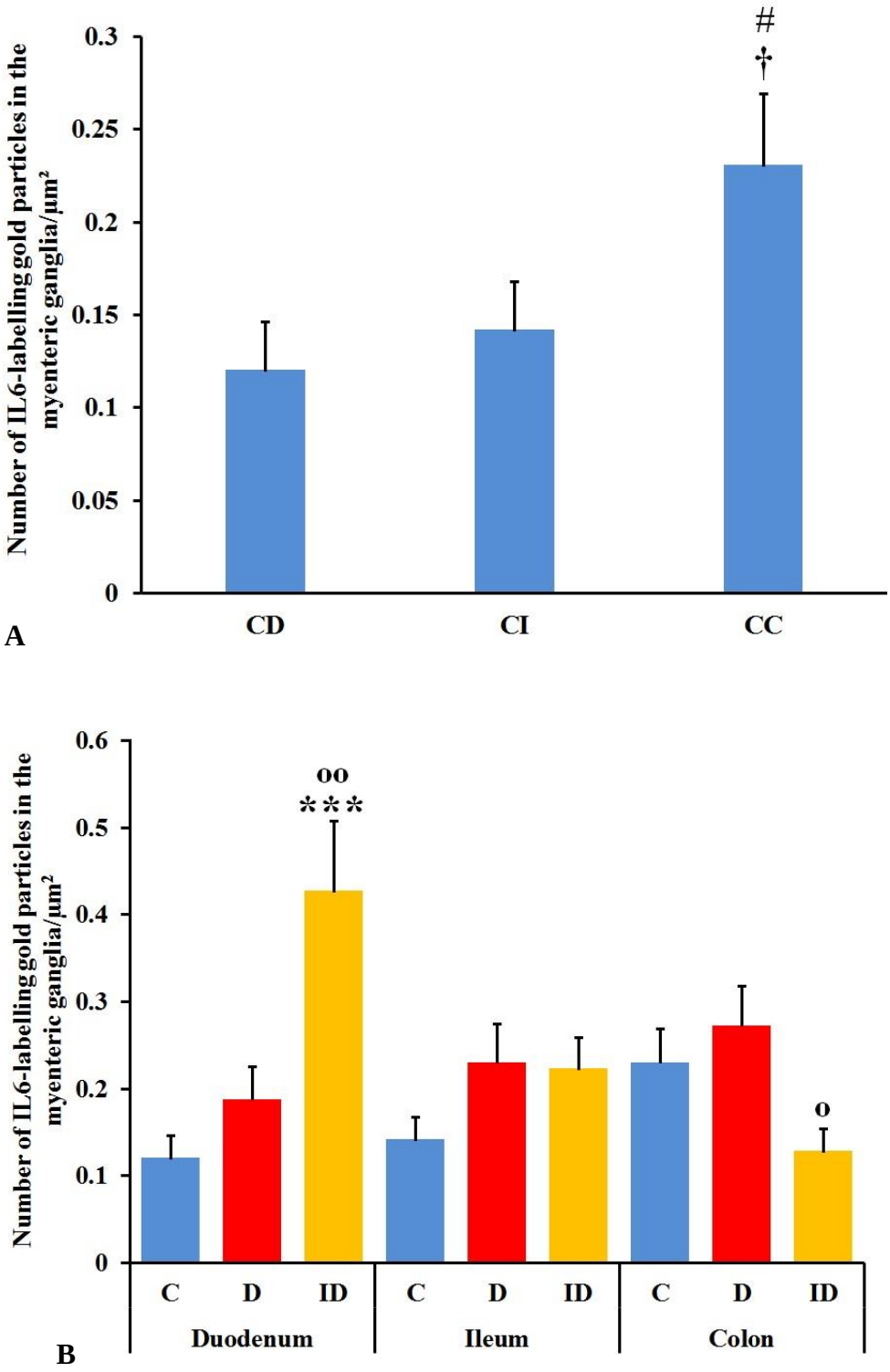


Figure 29. Number of IL6-labelling gold particles in the myenteric ganglia of different gut segments in controls (A) and under different experimental condition (B). Data are expressed as means ± SEM. $^{\dagger}P<0.05$ (compared to duodenum), $^{\#}P<0.05$ (compared to ileum); CD - control duodenum; CI - control ileum; CC - control colon.

*******P<0.001 (compared to controls); $^{o}P<0.05$, $^{oo}P<0.01$ (diabetics vs. insulin-treated diabetics). C - controls; D - diabetics; ID - insulin-treated diabetics.

Distribution of TNFα- and IL6-labelling gold particles in the capillary endothelium of different experimental groups

The number of TNFα and IL6 gold particles per unit area was counted in the capillary endothelial cells in the vicinity of the myenteric ganglia (Figure 30).

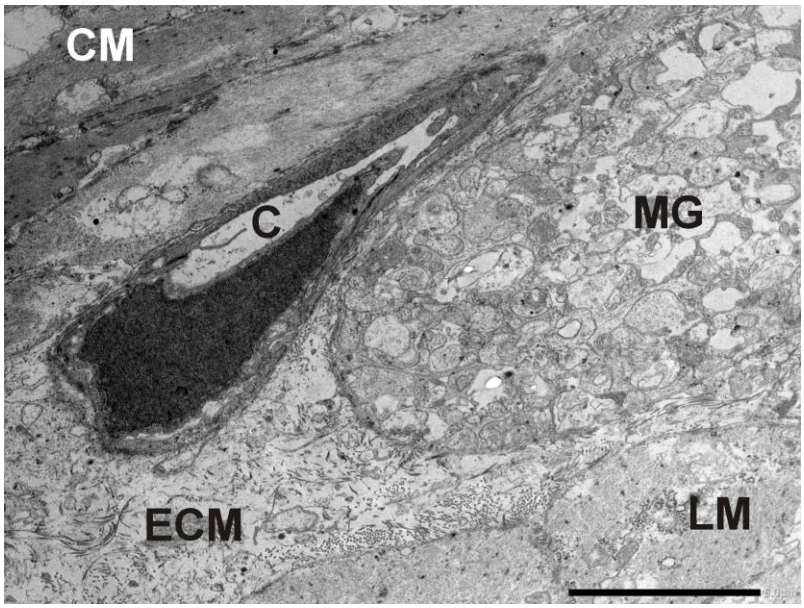


Figure 30. Representative electron micrograph of an intestinal capillary (C) adjacent to the myenteric ganglia (MG) in diabetic rat ileum. ECM - extracellular matrix, LM - longitudinal muscle, CM - circular muscle. Scale bar: 200 nm (insert).

The number of TNFα- and IL6-labelling gold particles were gut region dependent. The number of TNFα particles was distributed on the vesicle membrane, basement membrane and caveolae in the capillary endothelium (Figure 31).

In the controls, the number of TNFα-labelling gold particles was higher in the capillary endothelium of colon (0.82±0.18/μm^2) followed by duodenum (0.46±0.09/μm^2), and ileum (0.35±0.07/μm^2) (Figure 32A).

In diabetic rats, the TNFα gold labelling was slightly altered in duodenal (0.53±0.13/μm^2) and colonic (0.82±0.09/μm^2) segments, however, significant increase was observed only in the ileal (0.75±0.13/μm^2) capillary endothelium compared to control group. After immediate insulin treatment, the number of gold particles was elevated in the duodenum (0.65±0.16/μm^2) and ileum (0.60±0.11/μm^2), whereas it was decreased in the colon (0.40±0.06/μm^2) compared to control group (Figure 32B).

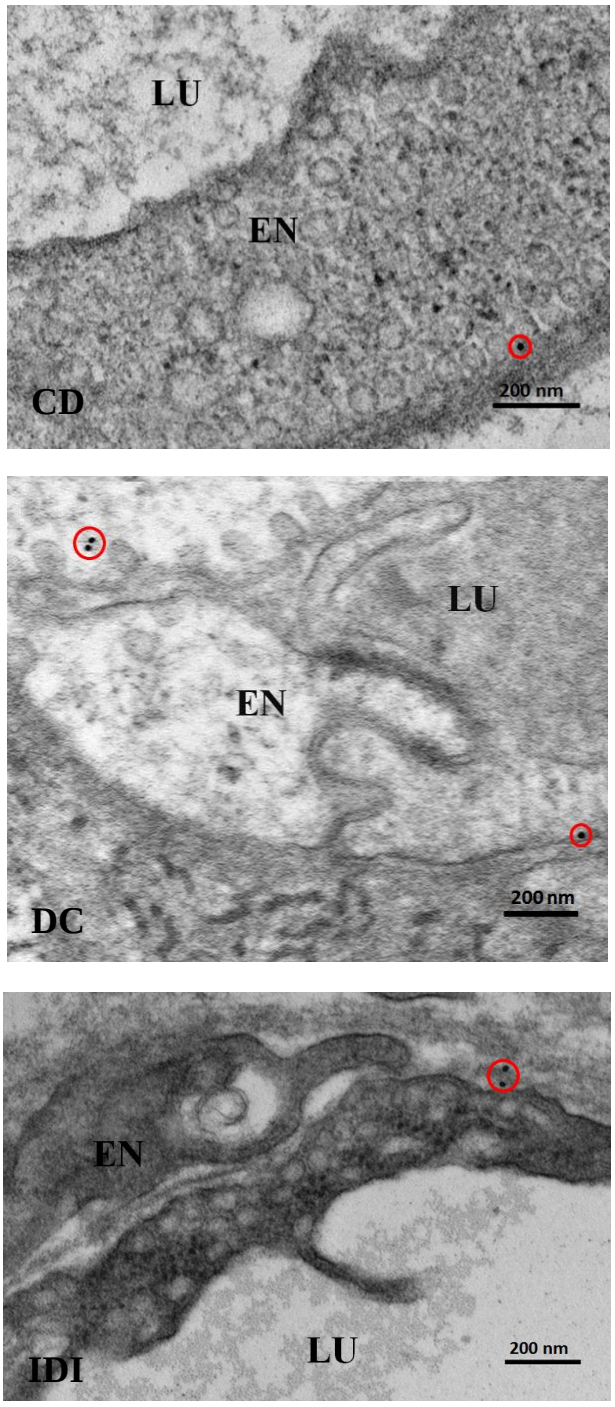


Figure 31. Representative electron micrographs of ultrathin sections of capillary endothelium from control duodenum (CD), diabetic colon (DC) and insulin-treated diabetic ileum (IDI). EN - endothelial cell; LU - lumen. The TNFα was labelled by 18 nm gold particles; shown in circle.

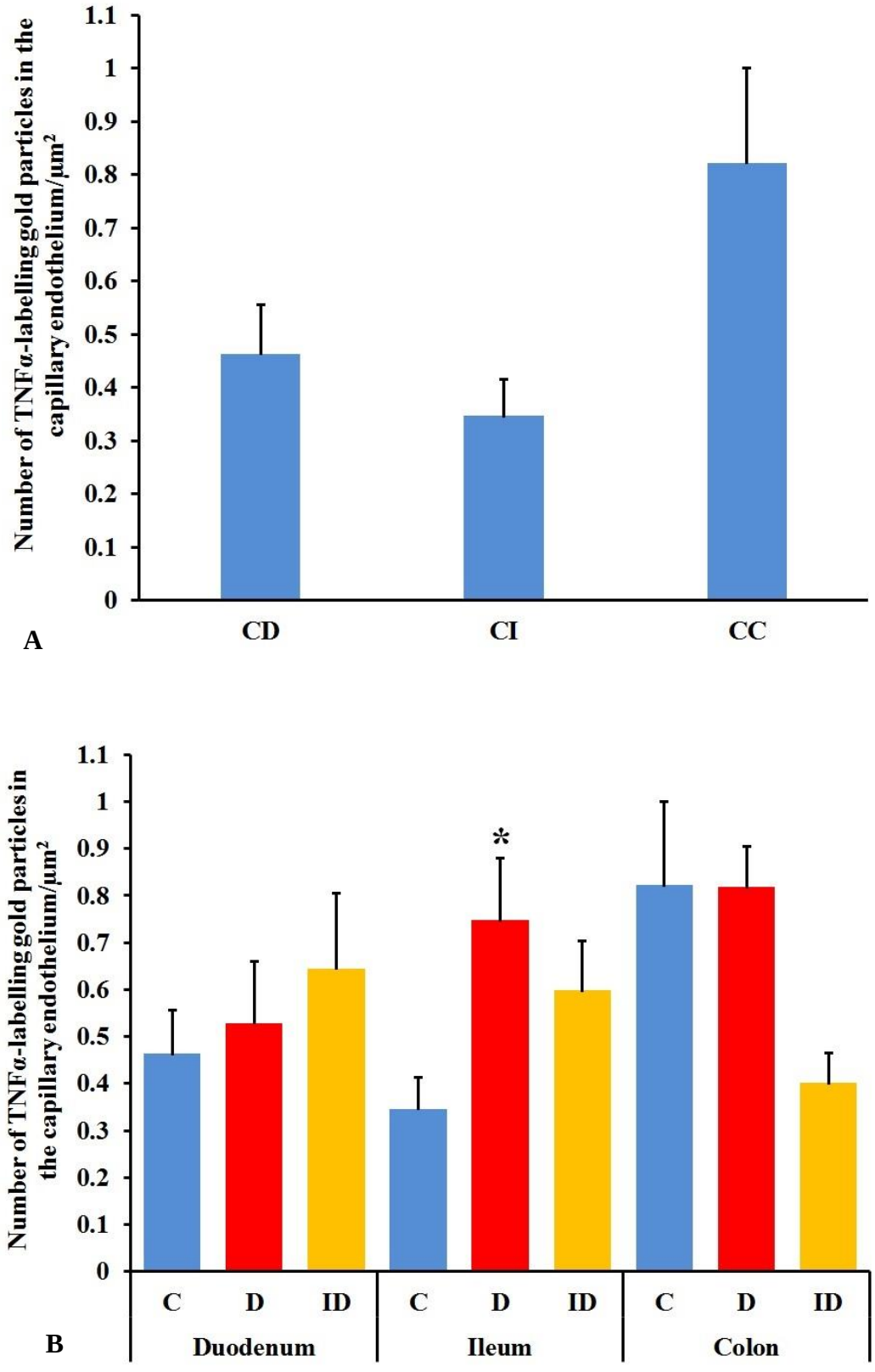


Figure 32. Number of TNFα-labelling gold particles in the capillary endothelium of different gut segments in controls (A) and under different experimental conditions (B). Data are expressed as means ± SEM. CD - control duodenum; CI - control ileum; CC - control colon.

*****P<0.05 (compared to controls); C - controls; D - diabetics; ID - insulin-treated diabetics.

The number of IL6 particles was distributed on the vesicle membrane, basement membrane and caveolae in the capillary endothelium (Figure 33). In controls, the expression of IL6 in the capillary endothelium was increasing to the distal part of the gastrointestinal tract. The number of IL6 gold particles was the highest in the colon ($0.55\pm0.23/\mu m^2$), followed by ileum ($0.13\pm0.09/\mu m^2$), and duodenum ($0.09\pm0.03/\mu m^2$) (Figure 34A).

In the diabetic group, the expression of IL6 was elevated ($0.58\pm0.21/\mu m^2$) in the duodenum and slightly increased in the colon ($0.32\pm0.10/\mu m^2$), whereas in the ileum the expression of IL6 was decreased ($0.11\pm0.03/\mu m^2$) without any significance compared to control group.

The immediate insulin treatment caused significant increase in the number of gold particles in the capillary endothelium of duodenal ($1.35\pm0.40/\mu m^2$), whereas in the ileal ($0.41\pm0.15/\mu m^2$) and colonic segments ($0.19\pm0.08/\mu m^2$) there were no significant changes between the insulin-treated diabetic and control group (Figure 34B).

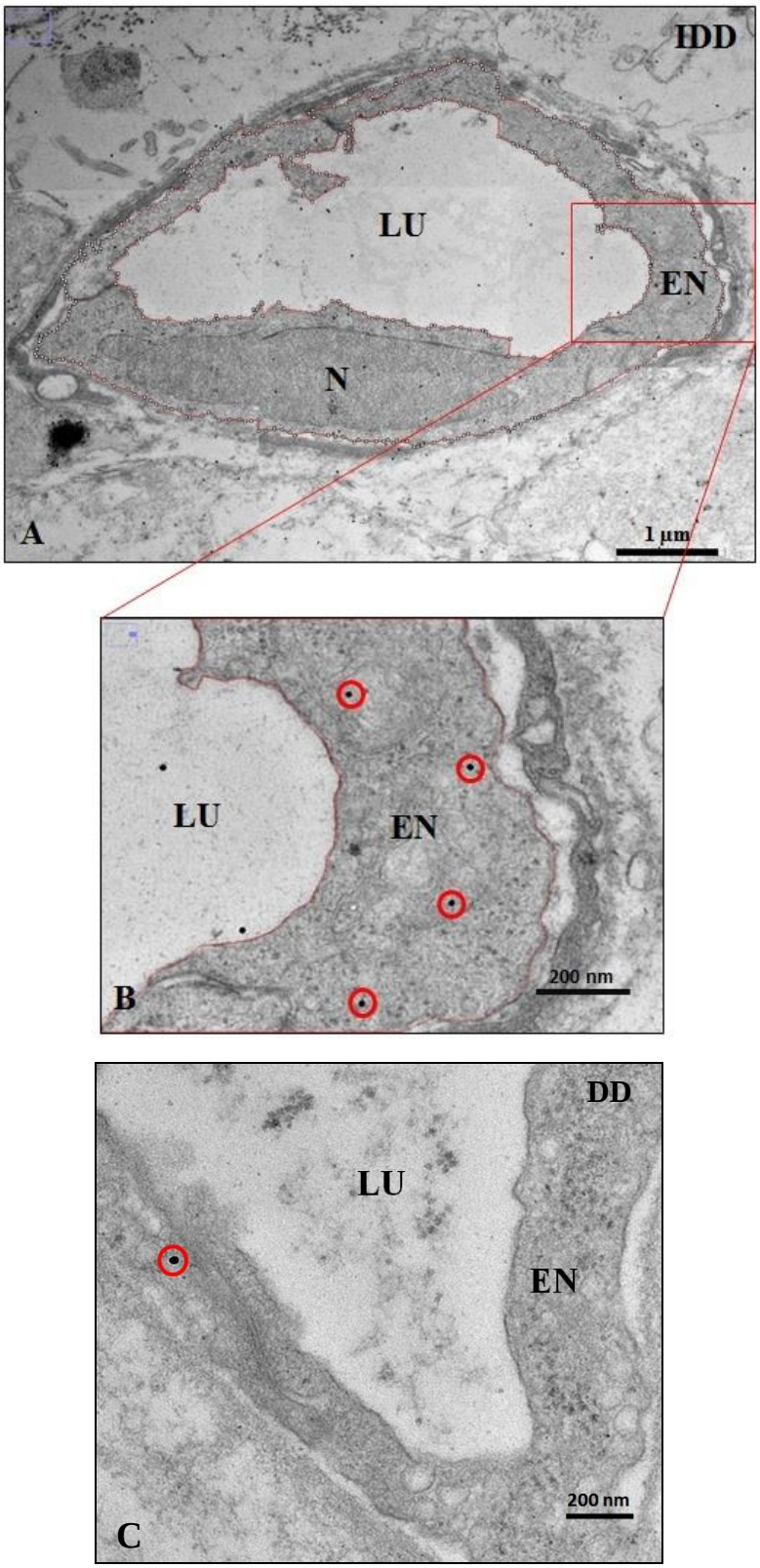


Figure 33. Representative electron micrographs of ultrathin sections of capillary endothelium from insulin-treated diabetic (IDD, A and B) and diabetic duodenum (DD, C). EN - endothelial cell; LU - lumen; N - nucleus The IL6 was labelled by 18 nm gold particles; shown in circle.

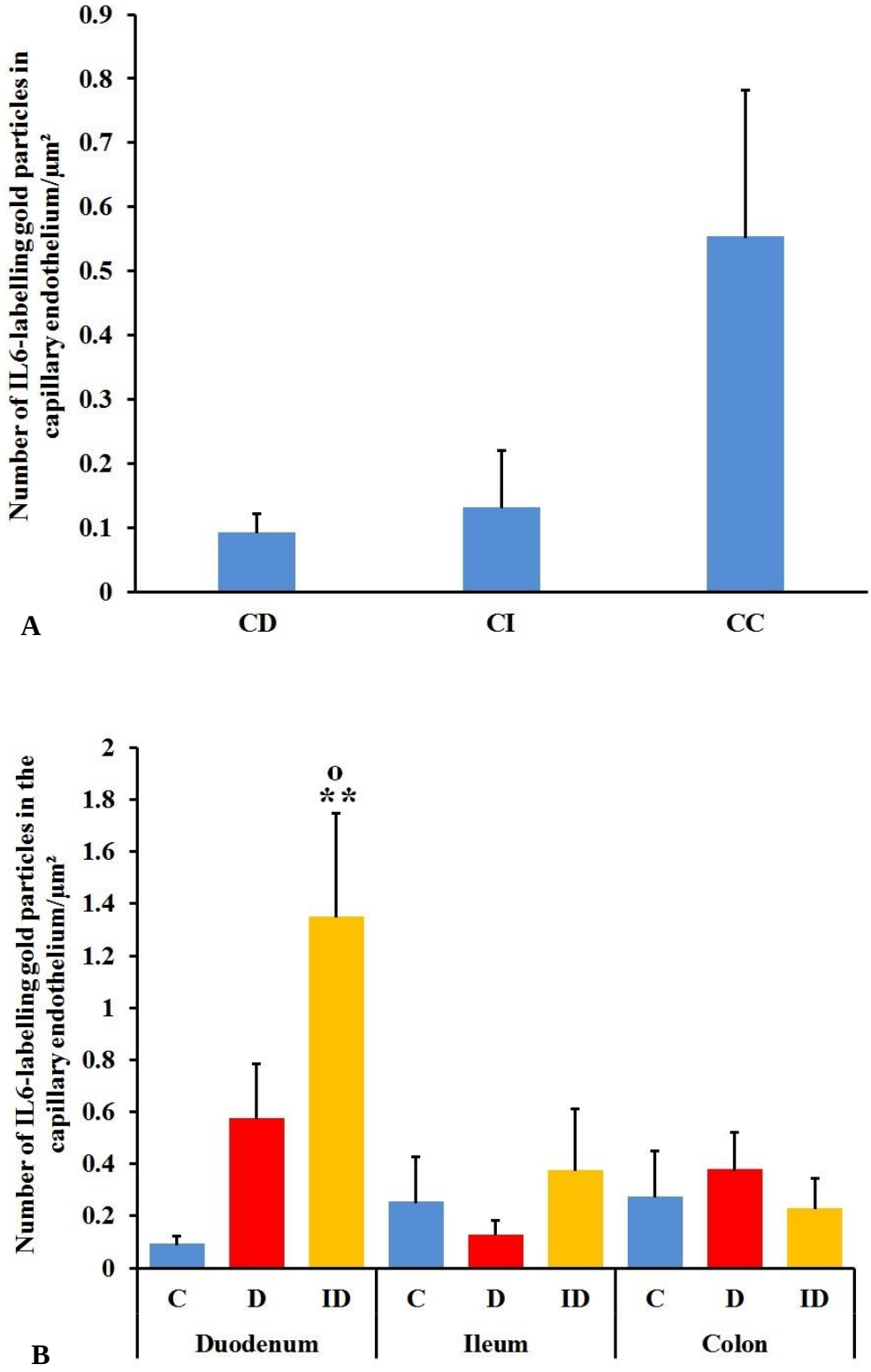


Figure 34. Number of IL6-labelling gold particles in the capillary endothelium of different gut segments in controls (A) and under different experimental condition (B). Data are expressed as means ± SEM. ******P<0.01 (compared to controls); ºP<0.05 (diabetics vs. insulin-treated diabetics). CD - control duodenum; CI - control ileum; CC - control colon; C - controls; D - diabetics; ID - insulin-treated diabetics.

5. Discussion

In support of our earlier finding that the susceptibility of nitrergic myenteric neurons by experimentally induced diabetes is strictly regional[3], our present study provides evidence of gut segment-specific diabetes-related induction of the endogenous HO system and also the intestinal-region dependent enhanced co-localisation of HO1 and HO2 with nNOS in myenteric neurons.

5.1 Distribution of HO1- or HO2-IR neurons and nNOS-HO1 or nNOS-HO2 co-localised neurons in the myenteric ganglia of control rats

The number of myenteric ganglia containing HO1- or HO2-IR neurons and those ganglia which include nNOS-HO1 and nNOS-HO2 co-localised neurons were more pronounced in the colon and low in the ileum of the control rats. Similarly, the number of HO1- or HO2-IR and nNOS-HO1 or nNOS-HO2 co-localised neurons were the highest in the colon and the lowest in the ileum under control conditions. Interestingly, in the duodenum of controls, although the number of HO1-IR or HO2-IR and nNOS-HO1-IR or nNOS-HO2-IR neurons were less than in the colon, still the presence of either HO-IR or nNOS-HO-IR neurons representing myenteric ganglia was nearly as explicit as in the colon.

Even under physiological conditions, these gut segment-specific differences raise the question of why the myenteric ganglia and nitrergic neurons are distributed differently, depending on their intestinal location. It is well established that the anatomical, functional, and pathological regionality of the GI tract develops under strict genetic control[144,145] which in itself can be responsible for the unique features of enteric neurons in a healthy state and for their different susceptibilities to pathological insults in different gut segments. It has been reported that alterations in gut microbiota[146] and redox imbalances are substantially linked to the GI disorders[147]. Emerging evidence also confirms that different degree of susceptibility of enteric neurons to a pathological stimulus such as hyperglycaemia might be related to the prevalence of bacteria in the different parts of the GI tract, which among others give rise to major differences in the intestinal redox status and the oxygen supply of the small and large intestine[148,149].

According to our results, in the controls, the colonic ganglia contains a high number of HO1- or HO2-IR and co-localised nNOS-HO1 or nNOS-HO2 neurons in the myenteric plexus. It has been reported that the colon was occupied with 90% of anaerobic bacteria and the microflora of facultative bacteria expands during infancy and creates a reducing

environment that supports the population of the gut by anaerobic strains, inducing a deep anaerobic state in the distal intestine[150,151]. The pro-oxidant environment of the colon compared to the small intestine may contribute to greater cancer susceptibility[152]. In our previous study[142], we demonstrated that gut region-specific accumulation of reactive oxygen species leads to regionally distinct activation of antioxidant and apoptotic marker molecules in proximal and distal part of the gut in rats with STZ-induced diabetes. There was no significant change in peroxynitrite level in the duodenum in any of the examined groups. However, in the diabetic colon, the peroxynitrite level was significantly increased, and the presence of severe necrosis was also confirmed by electron microscopy. These results also suggest that the distal part of the gut is more vulnerable than the proximal to oxidative stress. Therefore, we suppose that in the colon, where the baseline oxidative environment is far from optimal, the basal expression of HOs as essential players of the endogenous defense mechanisms is the most pronounced. In another study by Battish et al.[153] region-specific coexistence of HO2 with nNOS was also observed in the opossum anorectum, where the high percentage of co-localization in the myenteric plexus fell from almost 100% in the internal anal sphincter to 56% proximally in the rectum. This supports our findings that 44% of nNOS-IR neurons are also colocalized with HO2 in the colon of the control rats.

Further, it is noted that control ileum contained a low level of HO1- or HO2-IR and co-localised nNOS-HO1 or nNOS-HO2 neurons in the myenteric ganglia. This could be possible that ileum is more susceptible to the tissue damage in the intestine. It is in a good correlation with Umeda et al.[154] reported that HO1 protein was detected at low level in the ileal segment of mucosal epithelial cells and its expression was increased in haemorrhagic shock. This finding, further, adds evidence that little of any immunoreactivity of HO1 in the human antrum and jejunum[155]. We also found similar distribution in the myenteric ganglia of ileum. Moreover, Donat et al.[156] postulated that only 10% of HO-2 positive neurons also contained NOS in rat ileum which also supports our findings. Several studies also reported that HO2 proteins were distributed in different animal models such as in cat lower esophageal sphincter, in the mouse of small intestine and in the myenteric plexus of rat ileum[157].

We also assume that as a result of the adequate oxidative environment and definite baseline expression of HOs and their co-localization with nNOS in the myenteric ganglia of proximal small intestine, the nitrergic neurons get greater protection and could tolerate

hyperglycaemia-related oxidative stress better in the duodenum. This is supported by evidence[3] that despite the impairment of nNOS pathways in STZ-induced diabetic rats, the nitrergic myenteric neurons did not die in the duodenum, unlike the other gut segments. Other group also reported that highest density of HO2 neurons was observed in the duodenum of youngest fetal pigs[158]. Emerging evidences also reported that expression of HO2 immunoreactivity was highly observed in the myenteric neurons of the human stomach[159] and normal pylorus[160].

Moreover, the highest level of HO1 and HO2 expression were observed in the tissue homogenates of the control duodenum (including the smooth muscle layers of the gut wall and the myenteric plexus between them) which also highlighting an intensified protective environment in this particular gut segment and emphasizes the importance of the neuronal microenvironment[6,140]. Microsomal HO activity was also higher in the duodenal mucosa, where absorption of haemoglobin iron is reported to be most effective, and progressively fell in more caudal intestinal segments[161].

5.2 Distribution of HO1- or HO2-IR neurons and nNOS-HO1 or nNOS-HO2 co-localised neurons in the myenteric ganglia of diabetic rats

In diabetes, the number of myenteric ganglia containing HO1- or HO2-IR neurons and those ganglia that contain nNOS-HO1 and nNOS-HO2 co-localised neurons were intensely increased in the ileum. Likewise, the number of HO1- or HO2-IR and nNOS-HO1 or nNOS-HO2 co-localised neurons were markedly increased in the ileum and its expression differed from segment to segment in diabetic rats. Overall, the greatest alteration was observed in the ileum of diabetic rats which is considered to be the most concerned intestinal region in diabetes-related damage. Recently, we have reported[141] that ileal luminal samples from diabetic rats exhibited striking alterations in the gut microbiome, where the massive *Klebsiella* sp. invasion constitutes 31% that could directly associate with the pathological microenvironment. It is also reported that intestinal HO1, induced by the enteric microbiota modulates macrophage-mediated bactericidal activity, suggesting its importance in maintaining homeostasis[162]. On this basis, we suggest that the diabetes-related massive changes in the composition of the ileal gut microbiota[141] may contribute to the enhanced mucosal immune response and the greatest induction of endogenous HO system in this particular gut segment.

As regards the details of segment-specific diabetic alterations, the diabetic ileum containing both the HO1-IR and the nNOS-HO1-IR neuronal number were enhanced 7-fold, and the number of nNOS-HO2-IR neurons was also increased 6-fold as compared to controls. Although the total number of nNOS neurons was decreased in all intestinal segments of diabetics, confirming our earlier findings[3]. Therefore, we suggest that the high number of nitrergic neurons start to produce HO enzymes; further, those nNOS-positive neurons which are not colocalized with HOs will be destroyed during diabetic state. Similar alterations were seen in the colon, where besides a 22% decrease in the nNOS number, a more than 50% increase was demonstrated in the number of nNOS-HO1-IR neurons, from which we assume that HO-containing nitrergic neurons has higher protection, whereas the others are heavily affected by diabetic damage.

In the diabetic duodenum, besides a decreasing number of nNOS neurons, the number of co-localized myenteric neurons did not alter significantly, leading to the same conclusion. However, based on the study of Izbéki et al.[3] in which the duodenum was the only gut segment, where the decrease of NADPHd-positive neuronal number was not followed by a decrease in the number of total myenteric neurons, we suppose that in the present study, the reduced number of nNOS neurons denotes the cell loss in the diabetic ileum and colon but a change in neurochemical character in the duodenum. Indeed, the neuronal microenvironment, like capillaries of the gut wall and intestinal microbiota of the diabetic duodenum were broadly unharmed[6,140].

In an STZ rat model, the nitrergic myenteric neurons that also express HO2 were more resistant to the effects of diabetes and less likely to undergo apoptosis[163]. In the diabetic ileum, this study revealed an increased size of neuronal cell body in nNOS-IR neurons, while HO2-IR neurons remained unaffected. Double-labelling studies confirmed that the diabetes-induced change in size was confined to nNOS-IR neurons that did not contain HO2. The induction of the HO system by agents (such as several redox signals, hypoxia, or endotoxin) has been suggested to be an initial event in the development of an adaptive response to oxidative stress and inflammatory events in type 1 and type 2 diabetes[39,56,164-166].

Induction of HO1 reduces diabetes-induced glomerular injury and apoptosis in hypertensive rats[167]. Other group also reported that induction of HO1 improves glucose metabolism and protects the diabetes-related alteration in the renal tissues[168]. Bacterial endotoxin induces HO mRNA accumulation[169] and directly stimulates HO activity in

macrophages and the liver[170]. Emerging evidence indicates that the upregulation of the HO system and related products increases pancreatic beta cell insulin release and reduces hyperglycaemia in different animal models[171]. The HO system is also upregulated in short term diabetes, leading to HO and NO interactions, which may modulate vascular function in the retina[172,173]. Cheng et al.[174] reported that hypoxia-inducible factor-1α target genes like HO also contribute to retinal neuroprotection. It has also been demonstrated that NO and NO donors are capable of inducing HO1 protein expression, in a mechanism depending on the de novo synthesis of RNA and protein. Thus, it is postulated that NO may serve as a signalling molecule in the modulation of tissue stress response[175].

5.3 Distribution of nNOS-, HO1- and HO2-IR neurons in the submucous ganglia

We also investigated the nitrergic subpopulation of submucous neurons similarly to our earlier study on the myenteric plexus[3]. The present study describes that the total submucous neuronal number is similar in the duodenum, ileum and colon in the controls, and remains unchanged in diabetes as well as after insulin replacement. This is in agreement with the observation of da Silva et al. study[176], however their investigations are limited to the ileum. Our data reflect that STZ-induced diabetes does not induce degenerative changes in the total population of submucous neurons, unlike our previous data about the myenteric plexus. Oxidative stress is known to be induced by hyperglycaemia in diabetes via the mitochondrial overproduction of RONS which leads to an imbalance between free radical production and antioxidant defense molecules or mechanisms[13,142,177]. We suggest, in accordance with the opinion of Lopes et al.[178] that submucous neurons may have greater resistance to RONS under diabetic conditions. In non-diabetic state, nitrergic myenteric and submucous neurons have been examined along the gut in different rodent species, and it has been described that the myenteric plexus has more nitrergic neurons as compared to the submucous plexus[179-181]. Using the same rat model of T1DM as Izbéki et al.[3] the gut segment-specific alterations in the two ganglionated plexuses can be compared. According to the investigation of the myenteric plexus in the controls, the ileum contained the lowest proportion of nitrergic neurons[3], but we found no region-specific differences in the submucous plexus in accordance with other findings[182,183]. We have revealed different effects of hyperglycaemia on the ratio of nitrergic neurons in the two plexuses; a significant decrease was seen in all segments of the myenteric plexus, but, with the exception of the duodenum, an increase was found in the

ileal and colonic submucous ganglia. Our results demonstrate that not only the total neuronal density, but also the nitrergic subpopulation of the two plexuses are affected in a different way by the diabetic state. Because of the unchanged total neuronal number in submucous ganglia, alterations of the neurochemical character as an adaptation to diabetes-related oxidative stress is suggested to be in the background of these modifications. Neurochemical changes as an answer of the ENS to pathological conditions includes Crohn's disease, ulcerative colitis or Parkinson's disease are known[184-186]. Moreover, there is evidence that diabetic neuropathy and changes in neurochemical coding can occur in the submucous plexus of the ENS[178,187].

We found a similar result in another pathological rat model, in chronic ethanol-consumption, where the total myenteric neuronal number remained constant, but the density of nitrergic neurons was changed[28]. Previous studies showed that there are few nitrergic neurons in the submucous ganglia, the submucous plexus is more abundant in vasoactive intestinal polypeptide (VIP)-IR neurons as compared to the myenteric plexus, and there is a high proportion of VIP and nNOS co-localizing neurons in both plexuses[187,188]. Lin et al.[189] demonstrated that colonic VIP-IR submucous neurons start to express nNOS during culturing as a form of functional plasticity. Our results reported here show gut segment-specific alterations of not only the nitrergic, but also the HO1-IR and HO2-IR submucous neurons, and these alterations become increasingly pronounced along the proximo-distal axis of the gut. An important finding of this study is that HO1-IR and HO2-IR neurons are more abundant in the colon (about half of the colonic neurons was HO-IR) than in the small intestinal segments (about 0-5%) in the controls, and STZ treatment did not result in any significant changes in the investigated regions. The question arises as to what the reasons underlying these differences are. In non-diabetic state, more RONS are generated in the colon than in the small intestine[152]. It is possible that under worsening oxidative circumstances, the pro-oxidant basal state in the colon acts as a preconditioning factor inducing the higher physiological activity of both isoforms of the antioxidant HO enzymes. This mechanism may play a role in the protection of this segment against neuronal cell loss regarding the total submucous neuronal number in diabetic condition which is contrast to the earlier findings in the colonic myenteric ganglia.

The neuroprotective, antiapoptotic effects of the HO1 enzyme in oxidative stress were proved in the ENS[62,190]. However, we did not investigate the co-localization of nNOS with HO in these neurons, despite earlier findings demonstrating the beneficial effects of

the coexistence of these enzymes on the myenteric neurons[163]. Moreover, former studies visualized nNOS-HO2-IR neurons in the submucous ganglia[153,155,156] which suggests a protective role of HO, and may contribute to our results revealing the increasing proportion of nitrergic neurons in the diabetic ileum and colon.

The data about the effects of insulin are contradictory. While early insulin replacement was preventive to the total population of colonic myenteric neurons[3], we found no significant changes in the three intestinal regions in the total number of submucous neurons of insulin-treated diabetic rats. The density of nitrergic neurons showed similar patterns in the two plexuses after insulin replacement and increase was found from proximal to distal direction, which suggests significant changes in the ileum and colon as compared to untreated diabetic rats. As in the case of the nitrergic subpopulation, not the duodenal, but the ileal and colonic HO1- and HO2-IR submucous neurons responded to insulin treatment, but in an opposite way. However, there were no differences between the control and diabetic group in these two regions and we found an increased density in the ileum, but a decreased abundance in the colon of insulin-treated diabetic rats. Our results suggest that the three investigated gut segments have different levels of responsiveness to immediate insulin replacement in diabetes regarding the nitrergic and HO-IR subpopulation of submucous neurons. This is confirmed by our earlier findings in the myenteric neurons and in their microenvironment including the capillary endothelium adjacent to the myenteric ganglia[140]. The environmental changes of myenteric ganglia were described, such as the alteration of the faeces-associated microbiota in diabetes and insulin-treatment[141]. Moreover, microangiopathy as a complication of diabetes in submucous vessels of the GI tract was identified on duodenal and colon samples from diabetic patients[191,192].

On the other hand, our results are contradictory with the previous data about the beneficial or harmful effects of insulin. Insulin was shown to induce DNA damage through the enhanced production of RONS and to increase the risk of cancer in different cell types[193,194]. But insulin therapy decreased vascular superoxide overproduction in diabetic rats generated by NADPH oxidase, NOS or xanthine oxidase[195]. It is important to mention that the NO produced by NOS and the carbon monoxide generated by HO enzymes may have either pro-oxidant or antioxidant effects depending on the microenvironment, the duration of production and the amount of these gases[63,196-197]. This complex background makes it difficult to understand the details of our recent findings, and further experiments

are needed to explore the mechanisms of physiological and pathological events in the submucous neurons in diabetes and insulin replacement, their relationships and communication with their environment. Our present study provides evidence for the first time that the neurochemical character of the nitrergic submucous neurons exhibit gut-region dependent changes in diabetic and insulin-treated diabetic rats. In addition, we prove that HO1-IR and HO2-IR submucous neurons are present in small amounts in the small intestine, but in high abundance in the colon of control and diabetic rats, and they have segment-specific responsiveness to immediate insulin replacement.

In mouse models of diabetes, increased expression of antioxidants such as HO1 protected interstitial cells of Cajal from oxidative stress and reversed diabetic gastroparesis[51]. Attuwaybi et al.[198] reported that HO1 induction plays protective role against ischemia/reperfusion-induced injury of the small intestine by improved transit time and lessen mucosal damage. Recent discovery shows that HO gives neuroprotection by controlling redox formation and reducing production of TNFα[199]. In the light of these findings, the role of the endogenous HO system in the protection of intestinal motility in diabetics is further strengthened. However, the literary data suggest that TNF may also be capable of exerting opposite effects, which could depend on parameters such as the site, degree, and duration of the ischemic period, the amount of TNF production, the expression level of the two TNF receptors, and the cellular environment of affected neurons[94].

5.4 Distribution of TNFα- and IL6-labelling gold particles in the myenteric ganglia of different experimental groups

In this study, we quantified TNFα- and IL6-labelling gold particles in the myenteric ganglia and capillaries in the vicinity of them of control, diabetic and insulin-treated diabetic rats and provide evidence that distribution of these pro-inflammatory cytokines is strictly region-specific depending on the investigated gut segments.

The density of TNFα- and IL6-labelling gold particles was increased from proximal to the distal part of the GI tract of controls. Similarly, it is reported that neuronal expression of TNFα was varied in the different regions of murine brains under control condition[200].

Interestingly, the density of TNFα-labelling gold particles was significantly higher in the ganglia of diabetic and insulin-treated diabetic duodenum compared to the controls. This could explain that the myenteric neurons expressing this cytokine at high level in

order to protect them from the invasion of pathogenic microbes thereby preventing tissue injury in this particular gut segments. Our result is in correlation with Gonçalves et al.[201] reported that TNFα plays a significant role in controlling pathogenic bacterial populations and preventing tissue injury in the intestine of TNFRp55$^{-/-}$ mice. TNFα mediates its biological actions through TNF receptor mechanism: TNFR1 and TNFR2. It is reported, that TNFR2 signalling promotes neuronal survival and regeneration in the central nervous system of *in vivo* ischemic model[93].

Gougeon et al.[90] also reported that in a rat coculture model, TNFα and IL1β did not affect neuronal number, but promoted significant neurite outgrowth from enteric neurons. In accordance with these results and based on our earlier findings, we suggest that increased number of TNFα gold particles may play protective role in the duodenum via activating TNFR2 signalling pathway. Further investigation is needed to explore the TNFR signalling mechanism and the region-specific differences in the distribution of TNFR1 and TNFR2 in the myenteric neurons. Christen et al.[77] reported that expression of TNFα plays a dual role in T1DM, the study indicated that islet-specific expression abrogates the ongoing autoimmune process when induced late, irreversible but not the early phase of T1DM pathogenesis.

The tissue levels of TNFα and IL1β (including the smooth muscle layers of the gut wall and the myenteric plexus between them) increased significantly in the tissue homogenates originated from the duodenum of diabetic and insulin-treated diabetic animals, while the tissue levels of TNFα and IL1β did not change in the ileum and colon.

In the ileum, the density of TNFα-labelling gold particles was slightly increased in the diabetics compared to the control group, while in the colon it was significantly decreased. It is in correlation with our previous results, we suggested that colon was the only segment, where the anti-apoptotic bcl-2 expression was increased and severe necrosis was also confirmed by electron microscopy in diabetic rats[142]. The density of TNFα-labelling gold particles was significantly decreased in the insulin-treated diabetics compared to the control group which indicates that immediate insulin treatment did not prevent the expressional changes of TNFα in the duodenum and colon. Intrestingly, tissue level of TNFα and IL1β were unaltered in the ileal and colonic myenteric plexus of different experimental groups, while the tissue level of TNFα in the submucous samples decreased significantly in the ileum and colon compared to the controls. Surprisingly, the density of IL6-labelling gold particles was unaffected by diabetes in the myenteric ganglia

of duodenum, ileum, and colon. It is reported that IL6 protects pancreatic islet beta cells against pro-inflammatory cytokine-induced cell death and functional impairment in both *in vitro* and *in vivo* studies[202]. It is also reported that IL6 expression delays the onsite of T1DM in NOD mice[203]. Our argument is in correlation with the results published by Hundhausen et al.[204]: IL6 expression was declined in the serum of patients with long-standing T1DM, however, its expression was higher during early stage of disease condition.

There is evidence that insulin stimulates IL6 gene expression in human subcutaneous adipose tissue[205]. Rotter et al.[206] also reported that IL6 induces insulin resistance in human 3T3-L1 adipocytes. Andriambeloson et al.[113] reported that chronic intraperitoneal or subcutaneous administration of IL6 prevented sensory nerve conduction velocity, electrophysiological impairment and motor dysfunction in STZ-induced rats. Several studies also reported that IL6 plays protective role in the ischemic, ischemia-reperfusion injury[116], oxidative stress[117] and intestinal injury by heat shock models[207].

The density of IL6 gold particles was enormously increased in the duodenum of insulin-treated diabetics compared to diabetic and control group. This change in line with our previous results that immediate-insulin treatment could prevent the diabetes-related alteration of the microcirculation in the duodenum, but was not effective in the distal gut segments.

In the ileum, the density of IL6-labelling gold particles was remained at same level in the myenteric neurons of the diabetic and insulin-treated diabetic group. From our previous results, we reported that nitrergic subpopulation was reduced in the ileum of diabetic rats[3]. Taken together, we suggest that IL6 did not play any potential role in the myenteric neurons of ileal segment. Similarly, in the colon, the density of IL6-labelling gold particles induced slight changes in the myenteric neurons of the diabetics compared to control group. Constrastingly, in the insulin-treated diabetic group, IL6 expression was downregulated in the myenteric ganglia of colon. It is reported that IL6 deficient mice causes traumatic injury in the central nervous system[115]. It is also reported that reduced IL6 expression, indicated as a good marker in undifferentiated carcinoma[208].

5.5 Distribution of TNFα- and IL6-labelling gold particles in the capillary endothelium of different experimental groups

Similarly, our results according to the myenteric ganglia, the density of TNFα- and IL6-labelling gold particles in the endothelium showed segment-specific differences along the GI tract.

The density of TNFα-labelling gold particles was slightly increased in the capillary endothelium of diabetic duodenum compare to the control rats. Several studies have reported that TNFα is a vascular permeability-increasing agent which activates number of cell adhesion molecules and chemoattractants[209-212]. However, in contrast to this notion, from our previous results, we reported that the capillary basement membrane and vascular permeability were unaffected in the duodenum of diabetic rats[140] and the present findings support that TNFα did not mediate capillary inflammation in the duodenum of diabetic rats. This is consistent with Nilsson-Ohman et al.[101] reported that STZ-induced TNFα-deficient apoE-/- mice developed more pronounced vascular inflammatory response compared to wild-type mice. Thus, this study indicates that TNFα does not mediate diabetic-induced vascular inflammation in mice and reveal an unexpected protective role for TNFα.

In the ileum, the number of TNFα gold particles was enormously increased in the diabetes compared to controls. It is in a good correlation with our previous study, where structural, functional, and molecular alterations were observed in the capillary endothelium of ileal diabetic rats[140], although the immediate-insulin treatment did not prevent the number of TNFα gold particles in the capillary endothelium. In the colon, the number of TNFα gold particles was remained at the same level in the capillary endothelium of diabetic compared to control group. However, the immediate-insulin treatment decreased the density of TNFα gold particles in the colon, but did not reach the control level. In contrast, from our previous results, we demonstrated that the capillary adjacent to the myenteric plexus was highly damaged in the colon of the diabetic rats.

The number of IL6-labelling gold particles was slightly increased in the capillary endothelium of duodenum, ileum, and colon of the diabetic rats. Interestingly, the immediate-insulin treatment caused significant alterations in the expression of IL6 only in the capillary endothelium of duodenal segment.

6. Conclusions

The present study demonstrates evidence of intestinal region-specific diabetes-related alterations in the expression of endogenous heme oxygenase system and pro-inflammatory cytokines in enteric neurons and its microenvironment. We summarize the most important findings of our study as follows:

- We provide evidence of gut segment-specific diabetes-related induction of the endogenous HO system and also the intestinal region-dependent enhanced co-localization of HO1 and HO2 with nNOS in myenteric neurons. We presume that those myenteric nitrergic neurons which do not co-localize with HOs are the most seriously affected by diabetic damage.
- It has been demonstrated that the neurochemical character of the nitrergic submucous neurons exhibit gut region-dependent changes in the diabetic and insulin-treated diabetic rats. We proved that HO1-IR and HO2-IR submucous neurons are present in small amounts in the small intestine, but highly abundant in the colon of control and diabetic rats, and they have segment-specific responsiveness to immediate insulin replacement.
- The present study revealed a strictly gut-region dependent expression of TNFα and IL6 in the myenteric ganglia and supplying capillary endothelium of control, diabetic and insulin-treated diabetic rats. The density of TNFα- and IL6-labelling gold particles was gradually decreased from duodenum to colon in the myenteric neurons of diabetic rats. The density of TNFα-labelling gold particles in the duodenal endothelium did not show significant differences in the different animal groups, while the density of IL6 was slightly elevated in diabetics, and significantly higher in the insulin-treated diabetic rats compared to the controls.

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9. Summary

Background

Enteric nervous system (ENS) is the intrinsic nervous system of the gastrointestinal (GI) tract which is mainly interconnected by two ganglionated plexuses: myenteric (Auerbach's) and submucous (Meissner's) plexus. Enteric neurons are able to regulate the GI tract functions such as peristalsis, blood flow, mucosal secretion and absorption, highly independently of the central nervous system. The nitrergic myenteric neurons are key regulators of peristalsis in the GI tract. The impairment of nitrergic neurons has been implicated in a wide range of GI disorders in human and animal models.

We have previously demonstrated that the nitrergic myenteric neurons located in different intestinal segments display different susceptibilities to diabetic damage and different levels of responsiveness to insulin treatment. These results indicate that molecular differences in the neuronal microenvironment along the GI tract plays essential role in the pathogenesis of diabetic nitrergic neuropathy. Recently, we have demonstrated that long-lasting effect of hyperglycaemia results in increased oxidative stress and decreased effectiveness of the endogenous antioxidant protection that plays a major role in the initiation of diabetes-related neuronal damage.

Aims

Therefore, in this study, we used a streptozotocin (STZ)-induced diabetic rat model to investigate the effects of chronic hyperglycaemia and immediate insulin replacement in the ENS and its microenvironment along the GI tract. Our aim was to to determine the intestinal region-specific alterations in the expression of the endogenous antioxidant system and pro-inflammatory cytokines through the following questions:

1.1. Is there any gut segment-specific difference in the number of myenteric hemeoxygenase1 (HO1)- and HO2-immunoreactive (IR) neurons in the control?

1.2. Is there any effect of diabetes on the HO-neuronal nitric oxide sysnthase (nNOS) co-localization in the myenteric plexus of the different intestinal segments?

1.3. Is there any effect of the diabetes on the serum and tissue level of HO1 and HO2?

2.1. Is there any segment-specific effect of diabetes and immediate insulin treatment on the proportion of the nNOS, HO1- and HO2-IR submucous neurons?

3.1. Is there any gut segment-specific difference in the expression of tumor necrosis factor alpha (TNFα) and interleukin 6 (IL6) in the myenteric ganglia and surrounding capillary endothelium of the control, diabetic and insulin-treated diabetic rats?

3.2. Is there any effect of the diabetes and insulin treatment on the tissue level of TNFα, interleukin 1 beta (IL1β) and IL6 in the tissue homogenates containing myenteric or submucous plexus?

Materials and methods

Adult male Wistar rats kept on standard laboratory chow and with free access to drinking water, were used throughout the experiments. For the 10-week chronic hyperglycaemic study the rats were divided randomly into two groups: STZ-induced diabetics and sex- and age-matched controls. Besides these two groups, for the investigation of the HO system in the submucous plexus and the expression of the pro-inflammatory cytokines, a third group, an insulin-treated diabetic group was also used. Gut segments of duodenum, ileum and colon were processed for double-labelling fluorescent and post-embedding immunohistochemistry as well as enzyme-linked immunosorbent assay (ELISA).

In the myenteric plexus, we quantified the number of HO1- or HO2-IR myenteric neurons and those myenteric neurons in which nNOS was co-localized with HO1 or HO2.

In the submucous plexus, we quantified the proportion of nNOS-, HO1- and HO2-IR neurons to the total number of submucous neurons.

Further, we quantified the density of TNFα- and IL6-labelling gold particles in the myenteric ganglia and the endothelium of capillary in the vicinity of the myenteric plexus.

We also measured the tissue concentrations of HO1, HO2, TNFα, IL1β and IL6 in the different intestinal segments by ELISA.

Results

According to our results, the number of both HO-IR and nNOS/HO-IR myenteric neurons were the lowest in the ileal and the highest in the colonic ganglia of controls; it increased the most extensively in the ileum and was also elevated in the colon of diabetics. Although the total number of nitrergic neurons decreased in all segments, the proportion of nNOS-IR neurons colocalizing with HOs was enhanced robustly in the ileum and colon of diabetics.

The total number of submucous neurons and the proportion of nNOS-, HO1- and HO2-IR subpopulations were not affected in the duodenal ganglia of control, diabetic and insulin-treated rats. While the total neuronal number did not change either in the ileum or colon, the density of nitrergic neurons exhibited a 2- and 3-fold increase in the diabetic ileum and colon, respectively, which was further enhanced after insulin replacement. The HO1- and HO2-IR submucous neurons were robust in the colon of controls (38.4-50.8%), whereas it was significantly lower in the small intestinal segments (0.0-4.2%). Under pathophysiological conditions, the only alteration detected was an increase in the ileum and a decrease in the colon of the proportion of HO-IR neurons in insulin-treated diabetic rats.

The density of TNFα- and IL6-labelling gold particles was strictly region-dependent, with increasing to the distal part of the GI of controls. In diabetic rats, the number of TNFα-labelling gold particles was significantly increased in the duodenal, decreased in the colonic myenteric ganglia, while it did not show any significant difference in the ileal ganglia. The number of IL6-labelling gold particles was not affected by diabetes in the myenteric ganglia of different gut regions. The diabetes-related alterations of TNFα- and IL6 expression were not protected by the immediate insulin replacement in any of the investigated intestinal segments. The TNFα gold labelling was slightly altered in the duodenal and colonic segments, however, significant increase was observed only in the ileum of the capillary endothelium of diabetic rats. In contrast, the density of IL6-labelling gold particles were not significant in capillary endothelium even under different experimental conditions.

Discussion

The present study demonstrates evidence for intestinal region-specific diabetes-related alterations in the expression of endogenous HO and pro-inflammatory cytokines in enteric neurons and their microenvironment in experimental diabetes. We summarize our most important findings as follows:

We provide evidence for gut segment-specific diabetes-related induction of the endogenous HO system and also for intestinal region-dependent enhanced co-localization of HO1 and HO2 with nNOS in myenteric neurons. We presume that those myenteric nitrergic neurons which do not co-localize with HOs are the most seriously affected by diabetic damage.

We demonstrate that the neurochemical character of the nitrergic submucous neurons exhibit gut region-dependent changes in the diabetic and insulin-treated diabetic rats. We show that HO1-IR and HO2-IR submucous neurons are present in small amounts in the small intestine, but highly abundant in the colon of control and diabetic rats, and they have segment-specific responsiveness to immediate insulin replacement.

The present study reveals a strictly gut-region dependent expression of TNFα and IL6 in the myenteric ganglia and supplying capillary endothelium of control, diabetic and insulin-treated diabetic rats. The density of TNFα- and IL6-labelling gold particle was gradually decreased from duodenum to colon in the myenteric neurons of diabetic rats. The density of TNFα-labelling gold particles in the duodenal endothelium did not show significant differences in the different animal groups, while the density of IL6 was slightly elevated in diabetics, and significantly higher in the insulin-treated diabetic rats compare to the controls.

Based on these results, we suggest that the regional induction of the intestinal HO system, as well as, TNFα and IL6 expression are strongly correlated with diabetes-related region-specific nitrergic neuropathy.

Összefoglaló

Irodalmi háttér

Az enterális idegrendszer (ENS) az emésztőtraktus falában található, két ganglionált hálózatból: a myentericus (Auerbach) és a submucosus (Meissner) plexusból épül fel. Az enterális neuronok a központi idegrendszertől független működésre is képesek, fő feladatuk az emésztőrendszer felszívó, szekréciós és motoros működésének szabályozása. Az emésztőcsatorna perisztaltikájának legfontosabb szabályozói a nitrerg myentericus neuronok. Ismert, hogy a nitrerg enterális neuronok többféle emésztőrendszeri megbetegedés során is károsodnak, emberben és különböző állatmodellekben is.

Munkacsoportunk korábban bizonyította, hogy a bélcsatorna különböző szakaszaiban található nitrerg myentericus neuronok eltérő érzékenységet mutatnak a diabétesz-okozta sérülésekre és az azonnali inzulinkezelésre is. Ezek az eredmények arra utalnak, hogy a neuronális mikrokörnyezet molekuláris különbségei az egyes bélszakaszokban jelentős szerepet játszanak a nitrerg myentericus neuropátia kialakulásában.

A közelmúltban leírt eredményeink szerint a hosszú ideig fennálló hiperglikémia fokozza az oxidatív stresszt és csökkenti az endogén antioxidáns védelem hatékonyságát, és így nagymértékben hozzájárul a diabétesz következtében kialakuló neuronális károsodáshoz.

Célkitűzés

Streptozotocin (STZ)-indukált 1-es típusú diabéteszes (T1D) patkánymodellben vizsgáltuk a krónikus hiperglikémia ENS-re és annak mikrokörnyezetére gyakorolt hatását. Célunk az volt, hogy meghatározzuk az endogén hemoxigenáz (HO) rendszer és a gyulladáskeltő citokinek expressziójának diabétesz-okozta bélszakasz-specifikus megváltozását. Munkánk során az alábbi kérdésekre kerestük a választ:

1.1. Kimutatható-e bélszakasz-specifikus különbség a HO1- és a HO2-immunreaktív (IR) myentericus neuronok számában?

1.2. Kimutatható-e a diabétesznek a HO-neuronális nitrogén monoxid szintáz (nNOS) kolokalizációjára való hatása a különböző bélszakaszok myentericus plexusában?

1.3. Kimutatható-e a diabétesznek a szérum és a bélfal szöveti HO1 és HO2 szintjére gyakorolt hatása?

2. Van-e a diabétesznek, ill. az azonnali inzulinkezelésnek hatása a nNOS, HO1- és a HO2-IR submucosus neuronok megoszlására?

3.1. Kimutatható-e a tumor nekrózis faktor α (TNFα) és az interleukin 6 (IL6) expressziójában bélszakasz-specifikus különbség a kontroll, a diabéteszes és az inzulin-kezelt patkányok myentricus ganglionjaiban és az azokat körülvevő kapillárisok endotheliumában?

3.2. Kimutatható-e különbség a kontroll, a diabéteszes és az inzulin-kezelt patkányok myentericus vagy submucosus plexusát tartalmazó szöveti homogenizátumok TNFα, IL1β és IL6 szintjében?

Eredmények

Mind a HO-IR, mint az nNOS-HO-IR myentericus neuronok száma az ileumban volt a legalacsonyabb, a colonban a legmagasabb a kontroll patkányok esetén. A diabéteszes állatokban pedig ezen értékek a legnagyobb mértékben az ileumban, de a colonban is jelentősen emelkedtek. Bár a nitrerg myentericus neuronok száma minden vizsgált bélszakaszban csökkent, a HO-t is tartalmazó nNOS-IR neuronok aránya jelentősen megemelkedett a diabéteszes állatok ileumában és a colonjában.

A duodenum submucosus plexusában az összneuronszám és a nNOS-, HO1- vagy HO-2-IR neuronok aránya nem változott sem a diabéteszes, sem pedig az inzulin-kezelt állatokban a kontrollhoz képest. Az ileumban és a colonban bár az összeneuronszám nem változott, de a nitrerg neuronok aránya rendre kétszeres és háromszoros növekedést mutatott, amit az inzulinkezelés tovább fokozott. A HO1- és a HO2-IR submucosus neuronok száma jelentős (38,4-50,8%) volt a kontroll colonban, míg a vékonybél vizsgált szegmentjeiben szignifikánsan alacsonyabb (0,0-4,2%). A HO-IR neuronok arányát tekintve a diabéteszes minták nem mutattak eltérést a kontroll értékekhez képest, az inzulin-kezelt csoportban az ileumban emelkedés, míg a colonban csökkenés volt megfigyelhető.

A TNFα-t és az IL6-t jelölő aranyszemcsék denzitása bélszakasz-specifikus növekedést mutatott a kontroll patkányok béltraktusában disztális irányban.

A diabéteszes patkányok myentericus ganglionjaiban a TNFα-t jelölő szemcsék denzitása szignifikánsan magasabb volt a duodenumban, alacsonyabb a colonban, míg az ileumban nem mutatott szignifikáns eltérést a kontroll értékekhez képest.

Az IL6-t jelölő aranyszemcsék denzitását nem befolyásolta a diabétesz egyik vizsgált bélszakasz myentericus ganglionjaiban sem. A TNFα- és az IL6 expressziójában

bekövetkező, diabétesszel összefüggésbe hozható változásokat nem védte ki az azonnali inzulinkezelés.

A kapilláris endotheliumban a TNFα-t jelölő aranyszemcsék denzitása csak kis mértékben változott a duodenumban és a colonban, ugyanakkor szignifikáns emelkedést mutatott az diabéteszes ileumban a kontrollhoz képest. Ezzel ellentétben, az IL6-t jelölő aranyszemcsék denzitása egyik vizsgált kísérleti csoportban sem változott meg szignifikánsan.

Megbeszélés

Eredményeink alátámasztották, hogy a diabétesz az endogén HO rendszer és a gyulladáskeltő citokinek expresszióját entericus neuronokban és ezek mikrokörnyezetében bélszakasz-specifikus módon megváltoztatja.

A legfontosabb eredményeinket az alábbiakban foglaljuk össze:

Bizonyítottuk, hogy a diabétesz bélszakasz-specikus módon befolyásolja a HO1/HO2 és a nNOS-kolokalizációját myentericus neuronokban. Feltételezzük, hogy azok a myentericus neuronok, amelyek nem termelnek HO-t nagyobb fogékonyságot mutatnak a diabétesz okozta károsodásokra.

Bizonyítottuk továbbá, hogy a nitrerg submucosus neuronok neurokémiai karaktere bélszakasz-specifikus módon változik a diabéteszes és az inzulin-kezelt állatokban.

Kimutattuk, hogy a HO1- és a HO2-IR submucosus neuronok kisebb számban vannak jelen a vékonybélben, ugyanakkor nagy arányban a colonban, mind a kontroll, mind a diabéteszes patkányokban, és ezek a neuronok az inzulinkezelésre bélszakasz-specifikus érzékenységet mutatnak.

Bizonyítottuk, hogy a TNFα- és az IL6 expresszió a myentericus ganglionokban és az azokat tápláló kapillárisok endotheliumában bélszakasz-specifikus megoszlást mutat a kontroll, a diabéteszes és az inzulin-kezelt csoportban is. A myentericus neuronokban a TNFα-t és az IL6-t jelölő aranyszemcsék denzitása csökkenő tendenciát mutat a duodenumtól a colon felé haladva a diabéteszes állatokban. A TNFα-t jelölő aranyszemcsék denzitása a duodenum myentericus ganglionjaiban nem mutat szignifikáns változást egyik vizsgált állatcsoportban sem, míg az IL6-t jelölő aranyszemcsék denzitása emelkedik a diabéteszes, és szignifikánsan magasabb az inzulin-kezelt csoportban a kontroll értékekhez képest.

Ezen eredményeinket összefoglalva, a diabétesz az endogén HO rendszer bélszakasz-specifikus indukcióját okozza, valamint a TNFα és az IL6 expressziója szoros összefüggést mutat diabétesz-okozta bélszakasz-specifikus nitrerg neuropátiával.

10. List of publications

Publications related to thesis

MTMT number: 10053168

Full papers

1. **Chandrakumar L**, Bagyánszki M, Szalai Z, Mezei D, Bódi N (2017) Diabetes-Related Induction of the Heme Oxygenase System and Enhanced Colocalization of Heme Oxygenase 1 and 2 with Neuronal Nitric Oxide Synthase in Myenteric Neurons of Different Intestinal Segments. Oxidative Medicine and Cellular Longevity, Article ID 1890512. **IF: 4.593**
2. Bódi N, Szalai Z, **Chandrakumar L**, Bagyánszki M (2017) Region-dependent effects of diabetes and insulin-replacement on neuronal nitric oxide synthase- and heme oxygenase-immunoreactive submucous neurons. World Journal of Gastroenterology 23(41):7359-7368. **IF: 3.365**

Oral/Poster presentations

1. **Chandrakumar L**, Mezei D, Bence P B, Szalai Z, Bódi N, Bagyánszki M, Diabetes-related alterations in the expression of the inflammatory cytokines, tumor necrosis factor alpha and interleukin 6 in the myenteric ganglia and its microenvironment of different intestinal segments, 25th UEG Week, Barcelona, Spain, Oct' 17 to Nov' 1, 2017.
2. Bódi N, **Chandrakumar L**, Szalai Z, Bagyánszki M, Diabetes-related induction of heme oxygenase system and enhanced co-localization of heme oxygenase 1 and 2 with neuronal nitric oxide synthase in myenteric neurons of different intestinal segments, 59th Congress of the Hungarian Society of Gastroenterology, Siofok, Hungary, 10-13 June, 2017.
3. **Chandrakumar L**, Mezei D, Barta P B, Szalai Z, Bódi N, Bagyánszki M, Diabetes-related expressional changes of the inflammatory cytokine, tumor necrosis factor alpha in the myenteric ganglia and its microenvironment of different intestinal segments, Joint meeting of National Physiological Societies, Serbia, 25-27 May, 2017.
4. Bagyánszki M, **Chandrakumar L**, Szalai Z, Mezei D, Márton Z and Bódi N. Diabetes-related alterations of the heme oxygenase system in enteric neurons of different intestinal segments, 26th Congress of Hungarian Diabetes Association, Szeged, Hungary, 19-22 April, 2018.

Publications not related to thesis

Full papers

1. Talapka P, Berkó A, Nagy LI, **Chandrakumar L**, Bagyánszki M, Puskás LG, Fekete É, Bódi N (2015) Structural and molecular features of intestinal strictures in rats with Crohn's-like disease. World Journal of Gastroenterology 22(22):5154-5164. **IF: 3.365**
2. **Lalitha C** and Sohnachandrapackiavathy A (2011) Biochemical effect of hydroalcoholic leaf extract of Plectranthus amboinicus on Benzidine Induced carcinogenesis in male albino rats. Global Journal of Biotechnology and Biochemistry Research 1(1):31-38.
3. Praveen Kumar P, Kumaravel S and **Lalitha C** (2010) Screening of antioxidant activity, total phenolics and GC-MS study of *Vitex negundo*. African Journal of Biochemistry research 4(7):191-195.

Oral/Poster presentations

1. Bagyánszki M, Wirth R, Maróti G, **Chandrakumar L**, Szalai Z, Bódi N, Kornél L K, Regionally distinct alterations in the composition of the fecal and the mucosa-associated microbiota in rats with streptozotocin-induced diabetes, 25th Congress of Hungarian Diabetes Association, Pécs, Hungary, 20-23 April, 2017.
2. Mezei D, Bódi N, Wirth R, Maróti G, Bagyánszki M, **Chandrakumar L**, Szalai Z, Szucsán B, Kornél L K, Regionally distinct alterations in the composition of the lumen- and the mucosa-associated microbiota in chronic ethanol-treated rats, FIBOK, Budapest, 26-28 March, 2018.