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INVESTIGATING THE SHORT-TERM EFFECT OF DIABETIC PERIPHERAL NEUROPATHY ON DIFFERENT NEURONAL SUB-CLASSES IN CIRCUMVALLATE PAPILLA IN RATS

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ABSTRACT

Background: Diabetes Miletus (DM) is a major chronic disease that is spreading at an alarming rate worldwide because of genetic and environmental factors. Diabetic peripheral neuropathy is one of the most common complications of DM, where one of its manifestations is the impaired taste perceptions.

Aim: This study was performed to assess the effect of Diabetes Miletus on the nerves, taste buds' synaptic junction and ganglion cells of Circumvallate papilla including their three neuronal sub-classes.

Methodology: 12 animals were used in this experiment. Diabetes type 2 was induced in 6 male Wistar rats by feeding them 10% fructose solution for 2 weeks, followed by a single dose intra-peritoneal injection of STZ (40 mg/kg body weight). After rats were confirmed diabetic, they were left for five weeks. The Circumvallate papillae were examined by histopathological, Histomorphometric, and immunological analysis. The protein levels of CGRP, AChE, NGF, SNAP-25, and NOS were evaluated.

Results: Histological results showed notable increase in gustatory epithelium thickness, diminished taste buds' number as well as elevation in inflammatory cells level in the diabetic group. In contrast to control group, experimental group showed a higher significant NOS values compared to control group at p=0.01. For CGRP, AChE, NGF and SNAP25; experimental group showed lower values compared to control group at $p < 0.05$.

Conclusion: The short-term STZ induced diabetes has a destructive effect on different neural elements of CVP in rat including taste buds, synaptic proteins, nerves, and neurons.

KEYWORDS: Diabetic Peripheral Neuropathy, Taste impairment, Cholinergic. Nitrergic, Peptidergic.

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INTRODUCTION

Diabetes mellitus (DM) is a chronic disease characterized by hyperglycemia that results from either insulin deficiency, known as type 1 DM, or resistance to the actions of insulin, known as type 2 diabetes mellitus (T2DM) [1, 2]. Globally, DM has become a major worldwide health concern that affects nearly 8.5% of the adult population. DM is increasing at an alarming rate where it is estimated to rise to 578 million by 2030.

As a result of poor dietary habits and high prevalence of obesity, younger population became more liable to diabetes these days [3]. DM is a disease that affects the nervous system which is manifested in a variety of clinical forms; diabetic patients are more likely to acquire sensory disorders and impaired taste perceptions [4]. Diabetic peripheral neuropathy (DPN) is the most widespread complication in diabetes, affecting more than 50% of long-term type two diabetes mellitus (T2DM) patients [5]. Streptozotocin (STZ) has been extensively used in animals as a model to study the complications of DM as it exerts selective destruction of pancreatic β-cells in islets of Langerhans, resulting in insulin deficiency and hyperglycemia [6].

Circumvallate papilla (CVP) is a unique gustatory structure, as it is rich in taste buds and the presence of an associate secretory apparatus composed of a specialized serous gland, known as the von Ebner gland (VEG). These taste buds have intrinsic neurons that play a role in chemoceptive events modulating the taste cells [7]. The ultrastructure of a ganglion in the CVP of rat have been described, which are situated in the connective tissue below the CVP and named the CVP ganglion [8].

Sbarbati et al. concluded that there are three different neuronal sub-classes detected in the CVP ganglion, which are nitrergic, peptidergic and cholinergic neurons. They concluded that a large part of the neurons were positive for enzyme Nitric oxide synthase (NOS) and vesicular acetylcholine transporter (VAChT), while axons were evidenced mainly by their content of calcitonin gene-related peptide (CGRP) [7]. Several studies have suggested that diabetes may lead to neuropathy affecting nitrergic myenteric neurons, central cholinergic neurons and peptidergic nerve fibers [9, 10, 11, 12].

Nitrergic neurons are nerve cells that mediate transmission by nitric oxide (NO) [10]. NO can be generated by three different isoforms of the (NOS) including neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). nNOS is expressed in peripheral neurons, which help in smooth muscle relaxation, and vasodilatation via peripheral nitrergic nerves [13]. Peptidergic fibers express two neuropeptides CGRP and substance P, which are essential in pain transmission, they respond to nerve growth factor (NGF) via the expression of TrkA receptor [12].

Numerous studies showed the dependence of dorsal root ganglion and sensory neurons on NGF for development, maintenance, and survival. NFG is believed to be affected in diabetic neuropathy (**14**). Meanwhile, Synaptosomal Protein of 25 kDa (SNAP25) protein is involved in the processes of membrane fusion and exocytosis associated with neurotransmitter release in taste cells. It was proven that SNAP-25 can be used as a marker for taste cells possessing synapses in CVP of rat [15].

Neuronal tissue damage due to long-lasting high glucose levels, leads to injury of sensory, motors, and autonomic nerves [16]. Therefore, the goal of the present investigation was to assess the effect of short-term DM on the nerves, taste buds synaptic junction and ganglion cells of CVP including the three neuronal sub-classes; using histopathological, Histomorphometric and immunological analysis. The protein levels of calcitonin gene-related peptide (CGRP), acetylcholinesterase (AChE), NGF, SNAP-25, and NOS were detected by enzyme-linked immunoassay (ELISA).

MATERIAL AND METHODS

Ethical approval

The present study was approved by the Institutional Animal Care and Use Committee (IACUC) - Cairo University (Approval code: CU III F 56 22). This research was done in compliance with the ARRIVE guidelines and regulations (https://arriveguidelines.org).

Sample size calculation

According to previous study Abdulmalek & Balbaa 2019 [17], a total sample of 12 rats (6 per group) were found sufficient to detect an effect size of 2.08 a power of 0.85, a two-sided hypothesis test, and a significance level of 0.05. Sample size was calculated using G*power program (version:3.1), Germany [18].

Animals

Twelve adult male Wister rats weighing 250 to 350 grams were used in this study. The animals were obtained from Faculty of Medicine, Cairo University and maintained in an air conditioned animal house under controlled room temperature 25±2°C with 12/12 h light/dark cycle and were allowed to powdered soft food and water. Rats were housed in standard polycarbonate cages (Pretty industries, Model: CR5) as four animals per cage. The welfare of the animals was assessed prior to, during and after the experimental period by the attending veterinarian according to the ethical protocols for animal treatment that were supervised by the animal facilities, Faculty of Medicine, Cairo University.

Blood glucose levels measurement

At the beginning of the experiments, the blood glucose level of all rats was measured using a Fine test™ Auto Coding Premium blood glucose meter (Osang Healthcare Co., Ltd, South Korea). After gentle handling of the animals, one drop from the tail vein was applied on test strip to measure the glucose level, rats with levels of 300 or more mg/dL were defined as DM rats. The blood glucose level was assessed again at 3, 7 to and 30 days.

Experimental induction of T2DM and animal grouping

Diabetes was induced after a 1-week of adaptation period, as previously described by Wilson and Islam [19], where drinking water was replaced with 10% fructose solution ad libitum for 2 weeks to induce insulin resistance, while the control group was given only drinking water. Intra-peritoneal injection of STZ (40 mg/kg body wt.) (Sigma, St. Louis, MO, USA) in (0.01 M) Citrate buffer (pH 4.5) at day 0 was administered to rats to induce partial pancreatic β-cell dysfunction. Normal group were administered the same amount of physiological saline to control the influence of injection stress on the animals.

A week after induction of diabetes, the non-fasting blood glucose level (NFBG) was determined in the blood collected from the tail vein of all rats. Animals with NFBG level higher than 300 mg/dl for 7 consecutive days after the STZ injection were considered as diabetic and selected for the study as the experimental group while the non-diabetic rats were used as control group throughout the investigation period as shown in table 1.

TABLE (1) Animal grouping and study design

Group	Status	Chemical used	Route of injection	Number of animals
Control	Non-diabetic	physiological saline (2 mL/mg body wt)	Intra-peritoneal	6
Experimental	Diabetic	STZ (40 mg/kg body wt)	Intra-peritoneal	6

Tissue specimens and preparation

The rats were sacrificed with sodium thiopental (90-100 mg/kg-I.P) 35 days after T2DM induction. Then the CVP was dissected from the base of the tongue of all rats. The CVP from three rats per group underwent histological and Histomorphometric evaluation and the CVP from the other three rats per group underwent ELISA testing for immunological analyses.

Histological and Morphometric analysis

To evaluate the histopathological changes and perform the Histomorphometric analysis, specimens of the CVP were fixed in 10% formalin for 48 hours, they were routinely prepared and embedded in paraffin, and then they were cut in coronal sections (7 mm-thick) serially by microtome for morphometric analysis by a LEICA RM2255 light microscope equipped with digital camera and image analysis software.

- Gustatory epithelial thickness was measured from the basal membrane to the granular layer and used to indicate proliferative activity in three non-overlapping microscopic fields per slide [20].
- The number of normal ganglion cells in each papilla was counted using the method of Sbarbati et al., 2002 [7]. Each section was examined and number of ganglion cells with clear outline and open-faced nuclei were counted directly using a light microscope with magnification of x100 for better evaluation. Cells in the region between two parallel lines passing through the core of the papilla were counted. Each ganglion cell was traced by serial sections to avoid counting the same cell more than once. Any ganglion cells with signs of degeneration or unclear outline were not counted.

ELISA

After samples collection, CVP homogenate was prepared; the tissue was placed in iced normal saline and frozen at -80°C. The frozen tissues were cut into small pieces and homogenized in 5 ml cold buffer 0.5 g of Na2HPO4 and 0.7 g of NaH2PO4 per 500 µl deionized water pH= 7.4 then centrifuged at 4000 rpm for 15 minutes at 4°C, and the supernatant was collected for parameters estimation. The protein levels of NOS, CGRP, AChE, NGF and SNAP25 were measured in the CVP tissue supernatants using a commercially available ELISA kit, according to the manufacturer's instructions (Cusabio Technology LLC, USA).

Statistical analysis

Data showed normal distribution using Shapiro-Wilk test. Independent t-test used to compare between control and experimental groups. One-way ANOVA used to for blood glucose level assessment to show the effect of groups and time followed by pairwise comparison with Bonferroni correction. A significant level was set at p=0.05. (IBM SPSS, Version 23, Armonk, NY, USA).

RESULTS

Fasting blood glucose level

The diabetic group exhibited elevated blood glucose levels with a significant increase compared to control one for all tested periods at $p<0.001$. Also, the diabetic rats demonstrated increased glucose level after 7 and 30 days compared to 3 days. Remarkably, none of the diabetic rats' blood glucose level dropped to normal before the time of sacrifice (35 days). For control group, insignificant difference resulted in glucose level over time at p=0.978. as shown in table 2.

Histological results

Control group showed normal CVP morphology covered by typical epithelial thickness with normal underlying connective tissue structure. CVP revealed normal taste buds (TBs) number; TBs revealed normal mature barrel shaped structure. TBs showed different taste cell types with prominent nuclei and were surrounded with deep narrow troughs. There were numerous blood vessels and few inflammatory cells in the underlying connective tissue (**Figure 1A**).

TABLE (2) Blood glucose levels in rats under fasting conditions in experimental and control groups.

		Control		Experimental		p-value
		Mean	SD	Mean	SD	
Blood glucose level	3 Days	83.7[a]	4.4	359.2[a]	114.3	<0.001
	7 Days	88.2[a]	5.9	503.0[b]	92.1	<0.001
	30 Days	91.0[a]	5.2	568.2[b]	27.3	<0.001
p-value		0.978		<0.001		

Different letter within each column indicates significant difference at p<0.05.

The CVP ganglion is located at the base of the papilla above the lingual muscles and VEG acini. It is formed of a collection of neural ganglion cells surrounded by Schwann cells and combined with nerve fibers. It lies adjacent to the central nerve trunk and the main blood vessels. Ganglion cells were characterized by a large cell body containing a large open-faced spherical nucleus (fried egg appearance), a prominent nucleolus and basophilic cytoplasm. Ganglion cells aggregations are enclosed within non-continuous peri-neural capsule; they were surrounded by large blood vessels and few inflammatory cells (**Figure 1B**).

Experimental group showed slight deformity in the general outline of the papillae with noticeable hypertrophy in gustatory epithelium and decrease in TBs' number, which appears swollen and elongated while some are short and immature (**Figure 2A**). There was apparent fibrosis of connective tissue with focal aggregation of inflammatory cells (mostly neutrophils and lymphocytes) that invaded the nerve fiber plexus. Some ganglion cells showed signs of degeneration with cytoplasmic hyalinization and absence of nuclear details (**Figure 2B**).

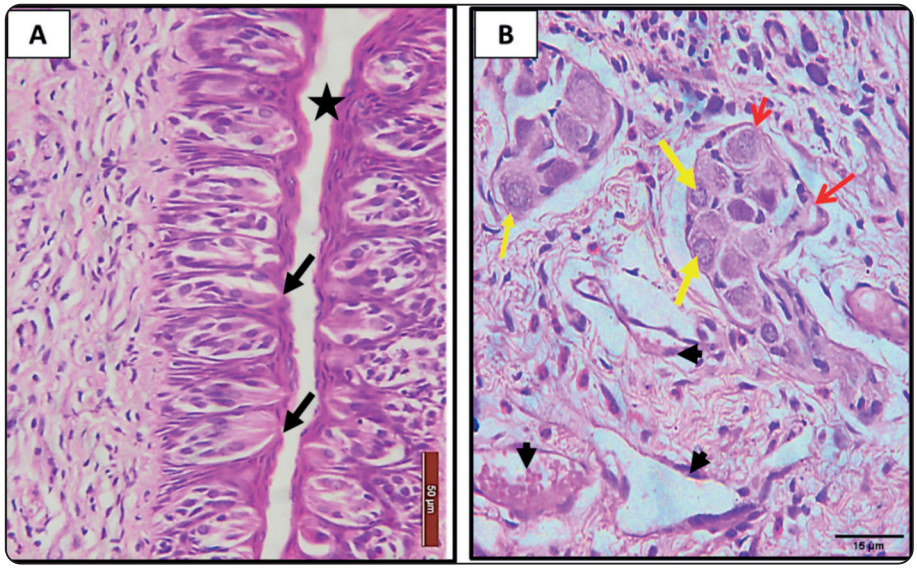

Fig. (1) Photomicrograph of control group; **1A:** showing normal gustatory epithelium with normal mature taste buds surrounded by deep narrow trough (Star) and taste pores (black arrows) (H&E, orig. Mag.X200). **1B:** Ganglion cells were characterized by a large cell body with a prominent nucleolus and basophilic cytoplasm (yellow arrows). Ganglion cells aggregations are enclosed within a non-continuous perineural capsule (red arrow), they were surrounded by large blood vessels (arrow heads) and few inflammatory cells (H&E, orig. Mag.X400).

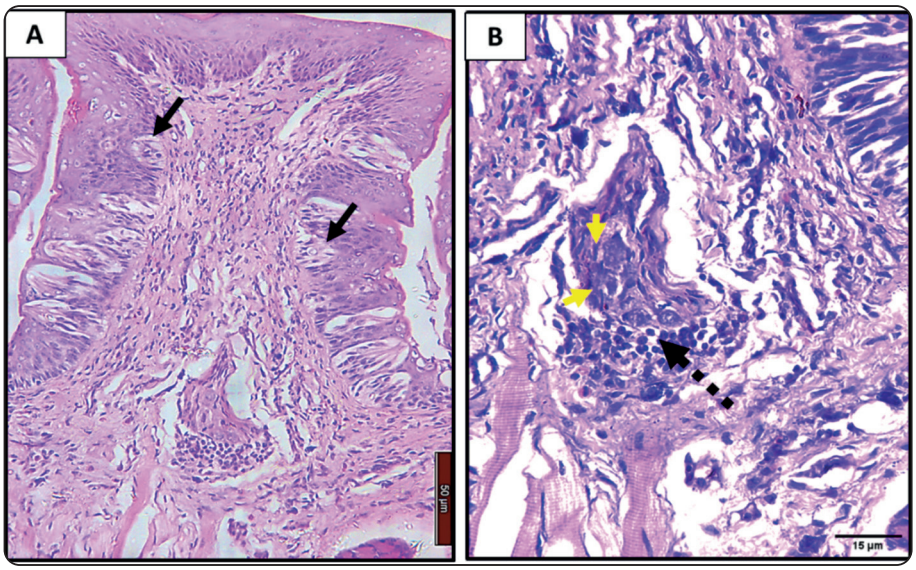

Fig. (2) Photomicrograph of experimental group: 2A showing visible increase in gustatory epithelium thickness and decrease in TBs' number, which appears swollen and immature (arrows). (H&E, orig. Mag.X200); 2B: There is moderate fibrosis with focal aggregation of inflammatory cells invading ganglionic nerve fibers plexus (dotted arrow). Some ganglion cells showed signs of degeneration with cytoplasmic hyalinization and absence of nuclear details (yellow arrow heads) (H&E, orig. Mag.X400).

Morphometric and quantitative analysis

Gustatory epithelial thickness and Ganglion cell count morphometric results

The epithelial thickness values were significantly higher in the experimental group compared to control group ($p<0.05$), and the ganglion cell count was decreased significantly compared to the control group (<0.001) (Table 3).

ELISA

Experimental group showed a higher significant NOS results compared to control group at p=0.01. For CGRP, AChE, NGF and SNAP25; experimental group showed significant lower values compared to control group at $p<0.05$ (Table 4).

TABLE (3) Histomorphometric assessment for the gustatory epithelial thickness and Ganglion cell count in control and experimental group

	Control		Experimental		95% Confidence Interval of the Difference		t	p-value
	Mean	SD	Mean	SD	Lower	Upper		
Gustatory epithelial thickness	253.3	32.1	461.1	61.8	-319.4	-96.1	-5.2	0.007
Ganglion cells count	31.0	2.6	11.0	2.0	14.7	25.3	10.4	<0.001

TABLE (4) Descriptive statistics and comparison between groups for NOS, CGRP, AChE, NGF and SNAP25 expression (ANOVA Test).

	Control		Experimental		95% Confidence Interval of the Difference		t	p-value
	Mean	SD	Mean	SD	Lower	Upper		
NOS (ng/ mg)	4.5	1.2	8.4	0.8	-6.1322	-1.5345	-4.630	0.010
CGRP (pg/mg)	118.0	4.6	86.3	4.7	21.115	42.219	8.332	0.001
AChE (U/mg)	2.0	0.2	1.1	0.2	0.4039	1.3294	5.200	0.007
NGF (pg/mg)	19.4	0.9	9.8	0.5	8.0240	11.1760	16.912	<0.001
SNAP25 (ng/mg)	6.0	0.7	4.0	0.3	0.8257	3.1743	4.729	0.009

DISCUSSION

The most prevalent complication of diabetes is DPN, as the nerve cells and their axons are so vulnerable to hyperglycemia. DM-related neuropathy affects nerve conduction, which affect neurotrophins-dependent innervation and impact taste perception. Numerous studies have shown taste impairment in T2DM, but the pathogenesis of such dysgeusia is still unclear [21,22]. This impairment may be due to micro- and macro-vascular complications of hyperglycemia, oxidative damage, reduced growth factor support and oral mucosal inflammation [22,23].

Due to the various etiologies of systemic and cellular disturbances in glucose and lipid metabolism, the current DPN prevention, diagnosis and treatment approaches are insufficient and ineffective. The most researched biochemical pathways are inflammation, altered Na+/K+ adenosine triphosphatase pump performance, and enhanced oxidative-nitrosative stress [24].

In the current study, the histological results showed that in comparison to control group, the experimental group had notable increase in gustatory epithelium thickness, reduced taste buds' number as well as elevation in inflammatory cells level with focal aggregation on the site of CV ganglion.

In spite that some studies have reported that hyperglycemia decrease epithelial proliferation [25], other studies reported the opposite. In accordance with the current finding, an increase in N/C ratio, cellular area, and cytoplasmic area was manifested in patients with T2DM, leading to cell swelling (cytotoxic edema) in the epithelial and glial cells [26]. Furthermore Tatiana *et al*. stated that diabetes enhanced intestinal epithelial cell proliferation in rats, they relates such change to β-catenin accumulation, which in turn activate the Wnt/β-catenin pathway [27].

In DPN, there is apparent elevation of nuclear factor-kappa light chain enhancer (NF-ϰB), which is implicated in production of pro- inflammatory cytokines. This is directly linked to increased oxidative nitrosative stress and inflammation. Inflammatory cytokines in turn can activate apoptosis, aberrant taste bud turnover, and taste bud loss. Moreover, increased levels of interluken-6 (IL-6), IL-1, tumor necrosis factor (TNF)-α and transforming growth factor-β (TGF- β) can cause nerve damage, reduced blood supply to nerves, and nerve apoptosis [24].

Recent findings suggest that the Wnt/β-catenin (concerned with development and tissue regeneration) and NF-ϰB (involved in inflammation) signaling pathways cross-regulate each of their activities and functions. Abnormalities within these two pathways result in a wide range of pathologies including inflammatory and metabolic diseases. Wnt/β catenin influences the activity of NF-ϰB signaling pathway either positively or negatively and vice versa [28]. So, a crosstalk between those two pathways may be implicated in the diabetic pathogenesis.

The aggregated inflammatory cells at the site of the ganglion that were noted in the present study were mostly neutrophils and lymphocytes. In experimental models of neuropathy, it was confirmed that there had been a sizable infiltration of neutrophils and macrophages at the site of the nerve injury. Additionally, it was discovered that persistent peripheral nerve injury results in neutrophil infiltration into the dorsal root ganglia (DRG) [29].

Likewise, when cytotoxic T lymphocytes attack a neuron, they release interferon gamma (IFN-γ), which is detected by the neighboring neuron through the appropriate receptor, activating and attracting nearby professional phagocytes (also known as "eating cells"). Phagocytes are then drawn in and engulf the synaptic connections with subsequent neurons degeneration [30].

In the ongoing study, different protein markers were selected to assess the degree of neural damage in nerves, taste buds and ganglion cells of diabetic groups using ELISA. One marker for each neuronal subpopulation was selected, (NOS) for detection of Nitrergic fibers, (CGRP) for Peptidergic fibers,

acetylcholine esterase (AChE) for Cholinergic structures. Also, Nerve Growth Factor (NGF) was selected as a neurotropic factor that supports the neurons' proliferation and survival, and SNAP25 as a marker for synapses in taste bud cells.

The results showed lower values of all investigated protein markers (CGRP, AChE, NGF, and SNAP25) in experimental group compared to control group except for NOS, which displayed a higher significant value compared to control group.

The investigation of the NOS enzyme activity in DRG of experimental diabetic models of rats of short (2 months) duration revealed an increase in total NOS activity in DRGs but there was no significant difference in mRNA expression of nNOS between diabetics and controls. This increase might resemble those that follow axotomy due to decrease of NO levels secondary to end products of glycosylate [31].

Meanwhile, DM effect on cardiac NOS expression were found to be dependent on the duration, where short duration (4 to 6 weeks) demonstrated an elevation of the total NOS activity as well as the mRNAs encoding eNOS and iNOS. In contrast, long duration (20 weeks) demonstrated reduction of NOS activity and the amounts of mRNAs of eNOS and iNOS [32].

NO activity has been postulated to be elevated early in diabetes but reduced in later stages of the disease [31]. Previous studies confirmed that nitrergic nerves undergo a degenerative process in two phases in PDN. During the early stages, the expression of nNOS is unaffected in the neuronal cell bodies but reduced in the nitrergic axons, probably due to a malfunction in axonal transport. nNOS begins to build up in cell bodies as DM progresses because it can no longer be delivered to the axons. The accumulation of advanced glycation end products in the blood and tissues occurs at the same time as increased nNOS protein and NO production [33].

Later, this enhanced oxidative stress results in cell bodies apoptosis. Accordingly, autonomic nerves may reach a threshold at which the degenerative changes become irreversible [33]. This could explain the rise in NOS levels in our short-term study and its decrease later with disease progression.

There is increasing evidence regarding the diminished expression of NGF and the dependent neuropeptides SP and CGRP in diabetes [14]. It was observed that decreased NGF content in early DM in some sympathetically innervated organs was followed by an increased NGF content in most peripheral targets in later DM (more than three months) [34]. Also, DM is associated with decline in neurofilament and peptide mRNAs which reflect defective uptake and transport of NGF [31].

In accordance with the current result, reduced expression of Snap25 was reported in T2DM, it was attributed to altered glycemic parameters and reduced exocytotic function [35]. Such a decrease might also be a result of the diminished number of mature taste buds.

The results herein highlight different possible explanations of the mystery behind the cause of taste impairment in diabetic patients. The associated inflammation and disturbance in main metabolic pathways linked directly to the subsequent damage in neural supply which is essential to gustatory apparatus maintenance. The defected synaptic junction proteins might be another etiology for the defected taste perception.

CONCLUSION

DPN in short-term STZ induced DM has a destructive effect on different neural elements of CVP in rat. The most evident histological observations were the prominent increase in gustatory epithelium thickness and diminished number of both taste buds and ganglion cells with focal aggregation of inflammatory cells in the site of ganglionic plexus indicating the implication of inflammation in the pathogenesis of taste impairment in DM. The immunological finding revealed significant lower values of cholinergic and

peptidergic neuronal sub classes markers indicating nerves and neurons degeneration. Alternatively, the nitrergic markers showed significant increase in comparison to normal, such elevation was concluded to be secondary to inflammation in relation to the disease onset. The NGF and SNAP25 expression was decreased in DM which reflects the adverse effect on taste buds' maintenance and function. Further studies are recommended to explore neural markers expression.

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