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ROLE OF GHRELIN ON CISPLATIN INDUCED MORPHOLOGICAL CHANGES ON SUBMANDIBULAR SALIVARY GLANDS

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ABSTRACT

Background: Cisplatin is a potent antineoplastic agent widely used for a variety of malignancies. However, it has many complications such as neuropathy and cachexia. Ghrelin is a recently discovered hormone with a potent orexigenic and neuroprotective properties that may prevent or reduce these complications.

Objective: This present study designed to determine the effectiveness of ghrelin administration as a protective agent against cisplatin-induced cytotoxicity in the submandibular glands of rats.

Design: Twenty –four adult male Sprague Dawley rats, were divided into three groups (n=8). Group (I) rats were received ghrelin 0.8 mg/kg twice daily for 14 day. Group (II) rats were received three doses cisplatin 0.5 mg /kg given daily. Group (III) rats were received cisplatin and ghrelin in dose as in group I, II. Daily measurement of body weight and blood glucose level. After 14 days, animals were scarified and the submandibular glands were removed and the prepared sections were examined histologically by H&E and immunohistochemical stain for inducible nitric oxide synthase (iNOS).

Results: Ghrelin administration in parallel with cisplatin-based chemotherapy were markedly stimulate appetite, with improvement of body weight and blood glucose level. Histological assessment revealed that ghrelin hormone reduce necrotic changes induced by chemotherapy.

Conclusions: Exogenous ghrelin markedly increase food intake and general nutritional health and also improve the induced cytotoxic changes during chemotherapy.

KEY WORDS: Chemotherapy, cisplatin, ghrelin, submandibular.

INTRODUCTION

Chemotherapy has been widely used for the treatment of malignant tumors and prior to radiotherapy and surgical intervention. It has cytotoxic effect on cancer cells and also affects the normal tissues, the amount of the damage and its severity is related to the type, amount and duration of drug used[1] .

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Mucositis, fungal infections, salivary secretion dysfunctions, and neuropathies are signs of early oral side effects [2] . Also; it leads to morphologic damage in salivary gland tissue [3] . Saliva is a fluid of the oral cavity produced by salivary glands with many functions. It acts as a first barrier against infections, so reduction in salivary flow may lead to the development of oral inflammation, increased dental caries[4] .

Cisplatin (CDDP) is a potent anticancer treatment widely used in various human neoplasms[3]. CDDP is a common deoxyriboneuclic acid (DNA) destructive agent, and it is mostly supposed that DNA platination is an important principal stage in its cytotoxic activity [5]. Cisplatin may directly stimulate the production of reactive oxygen species or may increase the release of reactive oxygen molecules usually synthesized inside mitochondria that may induce various pathway of apoptosis. Reactive oxygen species formed damage, which can arise as a result of antioxidant exhaustion and amplified lipid peroxidation [6] .

Ghrelin is secreted mainly by gastric endocrine cells, it is an endogenous ligand for the growth hormone (GH) receptor [7] . It improve food intake and prompt the nutritional health; and generate a positive energy equilibrium by a vital mechanism containing hypothalamic neuropeptides[8]. In rodents, cisplatin mostly reduced plasma ghrelin concentrations, while the administration of exogenous ghrelin reduce the cisplatin-induced effects in decrease the appetite and food intake [9, 10] .

Ghrelin synthesis occurs mainly in epithelial cells lining the fundus of the stomach, fewer quantities produced in the placenta, kidney, pituitary and hypothalamus [11] and human major salivary glands such as the parotid, submandibular, and sublingual glands [12] .

Nitric oxide (NO) is a gaseous free radical with a small biological half-life, which is formed enzymatically from L-arginine by a group of the NO synthase (NOS) isoforms [13] . Nitric oxide has been proposed to have a part in the formation of salivary amylase [14] , and induced protein secretion through an interaction with vasoactive intestinal peptide [15] . It has an significant role as a regulator of the salivary gland functions in physiological and pathological conditions [16] . It has a double performance in the regulation of salivary gland functions. It can plays as physiological messenger of numerous neurotransmitter receptors [17] or as an inflammatory mediator in a developing multiple of diseases [18] .

Accordingly, the present study designed to evaluate the influence of exogenous ghrelin administration as a protective agent against cisplatin-induced cytotoxicity in the submandibular glands of rats.

MATERIALS AND METHODS

Animals: Twenty –four adult male Sprague Dawley rats, weighting about 210 ± 30 gm took from animal house , Faculty of Medicine, Mansoura University, the rats housed in isolated metal cages under controlled temperature, humidity. They were kept on diet consisting of fresh vegetables, dried bread and tap water.

Material: Cisplatin was obtained from Sigma-Aldrich (St. Louis, MO), Rodent ghrelin was obtained from Phoenix Pharmaceuticals (Belmont, CA).Immunohistochemical stain for inducible nitric oxide synthase (iNOS)

Experimental procedure: The animals randomly divided into three groups:

Group (I): rats were received ghrelin 0.8 mg/kg twice daily by intraperitoneal injection for 14 day.

Group (II): rats were received three doses cisplatin. The dose of cisplatin was 5 mg / kg administrated daily from by intraperitoneal injection. This particular regimen of cisplatin was chosen because the total dose is similar to the dose of cisplatin administered to humans during a “cycle” of cisplatin [19] .

Group (III): rats were received cisplatin and ghrelin as in group I,II.

Daily measurements of body weight and blood glucose level were done by using blood glucose meter. After 14 days, all rats were scarified by ketamine over dose.

Specimen preparation: bilateral submandibular gland excision, then fixed immediately in 10% formalin. The section prepared to Haematoxylin and Eosin stain for routine light microscopic examination. And immunohistochemical staining of inducible nitric oxide synthase (iNOS).

Data obtained from body weight measurement, blood glucose level and inducible nitric oxide synthase (iNOS) expression were statistically counted in terms of mean ± standard deviation (± SD). One-way analysis of variance (ANOVA) test was used to compare between the groups followed by Tukey's post hoc test. A probability value (P-value) <0.001 was considered as highly significant and P-value ≤ 0.05 was considered significant. Statistical analysis was performed by Microsoft® Excel 2013 (Microsoft® Corporation, NY, USA) and Statistical Package for the Social Science (SPSS® Inc., Chicago, IL, USA) version 20.

RESULTS

1-Body weight:

Rats of group (I) had the highest body weight (246.33±10.29) while rats of group (II) had the lowest (135±8.41). Rats of group (III) (150±4.56) had body weights higher than those of group II but lower than those of control group.

Statistically, ANOVA test revealed an overall significant difference between all studied groups in relation to their body weights. Moreover, Post hoctukey test for multiple comparisons showed significant difference between group I on one hand and groups II, III other hand. There was a significant difference between group II on one side and groups III on the other side table (1) & Fig 1.

TABLE (1): Shows means± STD of the body weights of rats

	Group I	Group II	Group III	P
Mean ± STD	246.33± 10.29	135.00± 8.41	150.00± 4.56	**<0.0001**
P1	<0.0001	<0.0001	<0.0001	
P2			0.01	
P3			0.7	

STD: standard deviation P: Probability

Test used: ANOVA followed by post hoctukey for multiple comparisons

P1: Significance relative to Group I

P2: Significance relative to Group II

P3: Significance relative to Group III

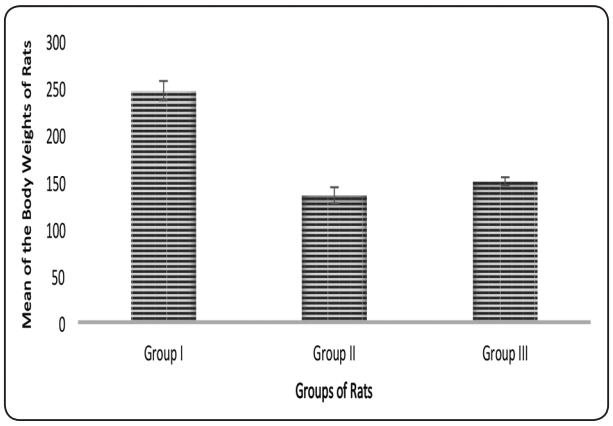


Fig. (1) Representing the body weights for different subgroups

2-Blood glucose level:

Rats of group I had the highest blood glucose level (115.83±2.11) while rats of group II had the lowest levels (67.92±4.78). Rats of groups III (105.25±3.93) had blood glucose level higher than that of group II but lower than that of group I.

Statistically, ANOVA test revealed an overall significant difference between all studied groups in relation to their blood glucose. Moreover, Posthoctukey test for multiple comparisons showed significant difference between group I on one hand and groups II, III on the other hand. Also, there was significant difference between group II on one side and group III on the other side table (2) &Fig 2.

TABLE (2): Shows means± STD of the blood glucose level.

	Group I	**Group II**	**Group III**	P
	115.83±2.11	67.92±4.78	105.25±3.93	
P1 **P2** **P3**	<0.0001	0.008 <0.0001	<0.0001	<0.0001

STD: standard deviation ***P: Probability***

Test used: ANOVA followed by post hoctukey for multiple comparisons

P1: Significance relative to Group I

P2: Significance relative to Group II

P3: Significance relative to Group III

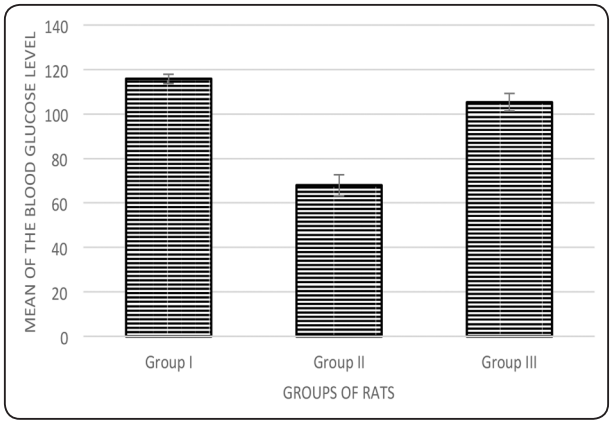


Fig. (2) Representing the blood glucose level for different subgroup.

3- Haematoxylin and Eosin (H&E) Stain

Group I: the sections showed normal histological and architectural features of rat's submandibular salivary gland Figs A (1,2).

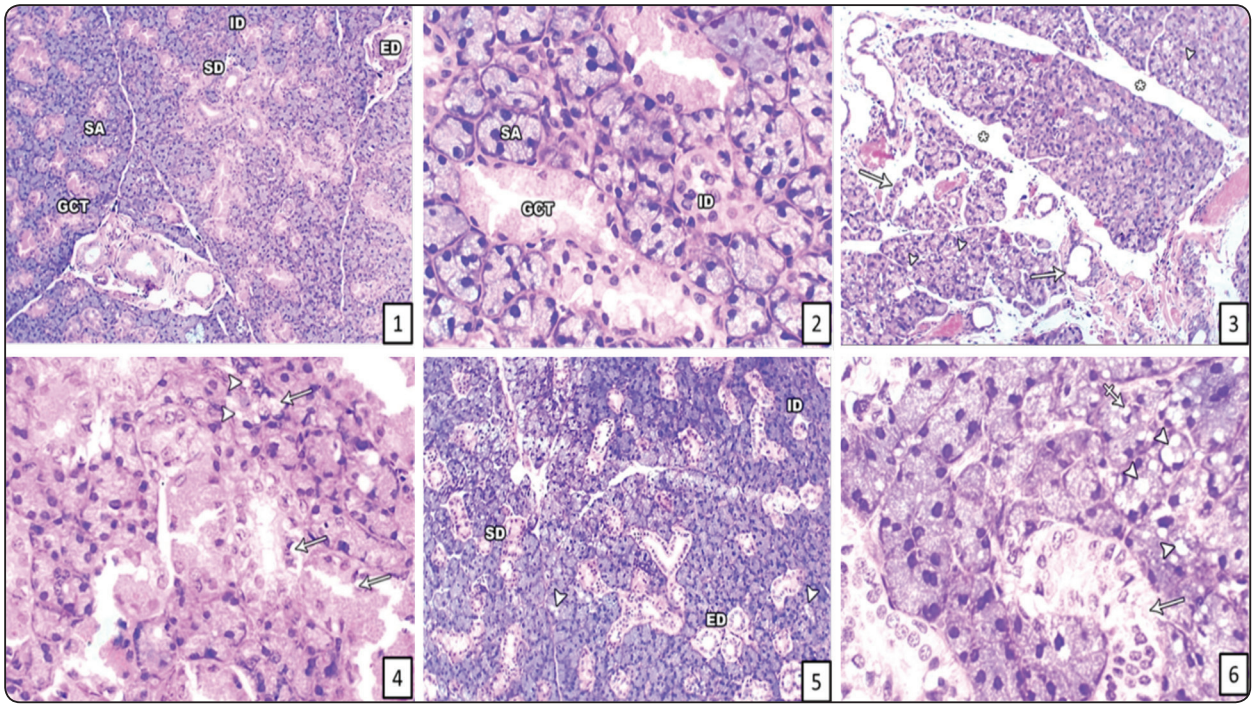
Group II: There were focal areas of loss of acinar and ductal architectural features, cytoplasmic vacuoles were prominent within acinar and ductal cells with necrotic changes as indented nuclei Figs A (3,4).

Group III: The submandibular salivary glands showed mild necrotic changes as cytoplasmic vacuoles and some shrunken indented nuclei were seen Figs A (5,6).

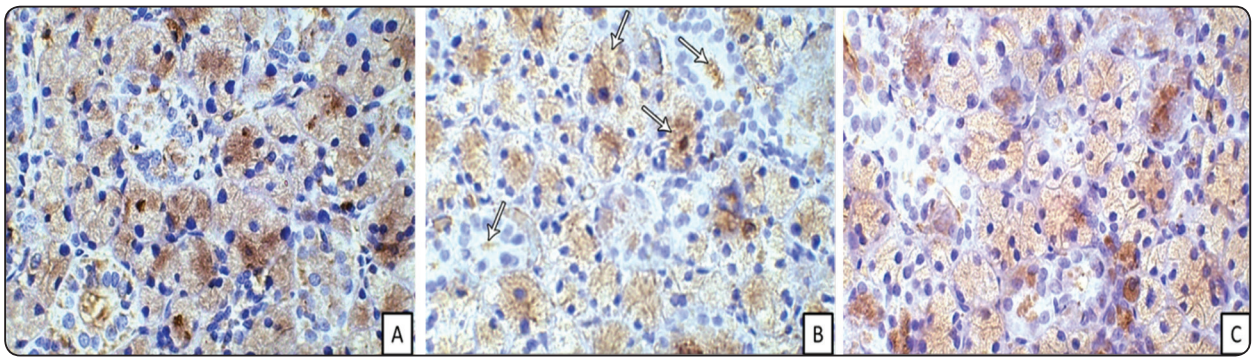
4- Immunohistochemical findings:

Cytoplasmic immunoreactivity for inducible nitric oxid synthase was detected in all studied groups with different levels. The highest level of iNOS expression was detected at group II while groups I, III had nearly the same level of iNOS expression.

Statistically, ANOVA test revealed an overall significant difference between all studied groups in relation to iNOS expression. Also, Post hoctukey test showed significant difference between group II on one side and groups I and III on the other side. While, there was a non-significant difference between group I on one hand and groups III on the other hand table (3) &Fig 3.



Figs (A): Group I (1) It shows lobular arrangement with appearance of densely packed serous acini (SA), striated duct (SD), excretory duct (ED), granular convoluted tubules (GCT) and intercalated duct (ID). (2) Serous acini (SA) formed of pyramidal cells with eosinophilic cytoplasm and spherical basally located basophilic nuclei, granular convoluted tubules (GCT) and intercalated duct (ID). Group II (3) slide showed degenerative changes of acini (arrow) and cytoplasmic vacuoles (arrowhead). Intralobular and interlobular spaces are wide (star). (4) Areas of focal loss of acinar and ductal structure (arrow), multiple intercellular vacuoles were seen (arrowhead). Group III (5) slide showed the glandular architecture of serous acini with some vacuoles (arrowhead), striated duct (SD), and intercalated duct (ID). (6) Loss of acinar outline and some indented nuclei (arrow) and cytoplasmic vacuoles (arrow head) (H&E X 100, X 400).



Figs B: (A) Photomicrograph of rat's submandibular salivary gland of group I immunolabelled with anti-iNOS showed mild positive reaction (15413314.00±219370.88) which appears as brown reaction within acinar and ductal cells (IHCX100). (B) Group II showed sever positive reaction (51451416.83±171866.78) within duct system and serous acini (IHCX100). (C) Group III showed mild positive reaction (15454123.67±152489.20) within duct system and serous acini (IHC X100).

TABLE (3): Showed mean ±STD of the iNOS expression

	Group I	Group II	Group III	
P	15413314.00 ± 219370.88	51451416.83 ± 171866.7	15454123.67 ± 152489.20	<0.0001
P1		<0.0001	0.97	
P2			<0.0001	
P3				

STD: standard deviation P: Probability

Test used: ANOVA followed by post hoctukey for multiple comparisons

P1: Significance relative to Group I P2: Significance relative to Group II

P3: Significance relative to Group III

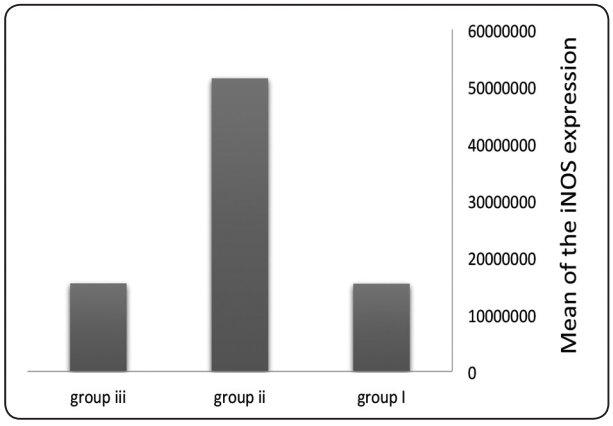


Fig. (3) Bar chart of (iNOS) expression for different group.

DISCUSSION

Cisplatin is a highly effective chemotherapeutic agent; it caused structural damage in the submandibular salivary gland and morphological changes in both acinar cells and the ductal system despite its potent antitumor effects[3].

The current study demonstrated that rats of group (I) had the highest body weight (246.33±10.29) while rats of group (II) had the lowest (135±8.41). Rats of group (III) (150±4.56) had body weights higher than those of group II but lower than those of control group. This is findings was in agreement with Warzecha Z, et al, who explained that ghrelin enhances food intake which is the most important physiological stimulator of salivary secretion[20]. Moreover, salivary glands secrete ghrelin and amount of ghrelin in saliva increases during meal intake[21]. Nausea and Anorexia have been considered the main toxicities described with cisplatin-based chemotherapy. Ghrelin administration significantly decrease these side effects to 15% and 20%, respectively[22] . Numerous observations recommend that ghrelin can form a vital role in the reduction of induced gastrointestinal complication during cisplatin treatment. In rodents, only a dose of cisplatin administration produced a temporary reduction of plasma ghrelin concentration and extended decrease in food consumption and body weight loss [23] . several observation confirmed that the treatment with exogenous ghrelin during chemotherapy positively improved nutritional health [22] .

The current study, the changes in rat submandibular salivary glands after cisplatin infusion were examined, and reported the focal areas of loss of acinar and ductal outlines, there were extreme degenerative and necrotic changes as indented nuclei and cytoplasmic vacuoles observed at acinar and ductal cells. Despite its potent anti-tumor effects, cisplatin is known to cause serious side effects. Kitashima reported that cisplatin-induced morphological changes in the submandibular salivary gland of rats, which were detected in both acinar cells and the ductal system [24] . Cisplatin can reduce saliva production in at least two ways: initially, through blocking of aquaporin expression, or by stabilizing DNA strands which especially prevents the regeneration of glandular tissue when progenitor cells are damaged [25] .

Ghrelin mRNA was detected in the parotid and submandibular glands, but was not noticeable in the sublingual glands and its proteins were prevalent in the cytoplasm of striated, intercalated and excretory ducts, in addition to the serous acini of parotid and submandibular glands, but not in the mucous acini of sublingual glands. So, the parotid and submandibular glands were principal sources of ghrelin formation and secretion in saliva [26] .

The cytoprotective effect of ghrelin as a mediator against cisplatin-induced cytotoxicity were examined in the rat submandibular salivary gland. The histological results showed reduced necrotic changes as cytoplasmic vacuoles and some shrunken indented nuclei, Ghrelin improve the appetite and displays some positive role on energy metabolism, mainly in anorexic disorders, specially a clinical application for patients receiving chemotherapy[27]. In addition to its orexigenic properties, ghrelin inhibits cell death in numerous tissues [28].

The numerous types of chemotherapy treatments trigger the production of free radicals, both in vitro and in vivo. Free radicals are known as reactive oxygen species with highly reactive with several cellular compounds, that could result in production of a cascade of oxidation and reduction reactions. This oxidative stress reaction, under definite conditions, might cause structural and functional alterations in healthy cells and organ dysfunction[29]. Ghrelin administration protects hypothalamic neuronal cells in an oxygen-glucose deficiency model by preventing the production of reactive oxygen species, which leads to preventing cytochrome c release, and caspase-3 activation [30] .

NO is compromised in controlling of several physiological processes, such as vascular relaxation, neurotransmission, immune regulation and cell death and created by nitric oxide synthase (NOS. also, NO is a component of saliva and is produced by oral bacteria in the oral cavity and released by NOS expressed in oral mucosa [31] . in addition, NOS was reported to have a part in the synthesis of salivary amylase and a controller of the salivary gland functions in physiological and pathological conditions [14, 16].

The immunohistochemical and statistical analysis for (iNOS), showed that the maximum level of iNOS expression was distinguished at group II while groups I, III had nearly the same level of iNOS expression. Nitric oxide (NO) is a short-lived signaling molecule that has an essential part in numerous of physiologic tasks, containing the regulation of blood vessel tone, inflammation, mitochondrial functions and apoptosis [32, 33]. Ghrelin possesses anti-inflammatory properties, which may counteract the pro-inflammatory response induced by cisplatin. Although it is not yet clear how ghrelin and its receptor are mediating the apoptotic response to cisplatin, ghrelin may be performing as a survival factor through the prevention of these apoptotic pathways and the protection of mitochondrial vitality [34] .

In conclusion, it has been reported that when ghrelin and cisplatin are working together, the long-term damage is reduced. These results recommend the possibility that clinical use of ghrelin could decrease or prevent damage to the salivary glands of patients receiving cisplatin chemotherapy. However, additional research and prolonged observation are required.

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