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RESEARCH ARTICLE

Nesfatin-1 treatment alleviates indomethacin-induced gastric ulcer in rats

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Abstract

Background: Indomethacin is a non-steroidal anti-inflammatory drug, which is known to produce serious adverse effects, including ulcerative lesions. Nesfatin-1, a newly identified anorexigenic peptide, was recently shown to have antioxidant effects.

Aim: This study aimed to investigate the possible ameliorative effect of nesfatin-1 against indomethacin induced gastric ulcer in rats, with exploration of the possible mechanisms underlying this effect.

Design: 24 adult male albino rats were used. Ulcers were induced using oral indomethacin (20mg/kg) daily for 7 days. The rats were randomly assigned to vehicle treated control group(I), indomethacin treated (II), and indomethacin treated for 7 days followed by nesfatin-1 (1ug/kg i.p) daily for 10 days. Assessment of gastric juice parameters (total acid output, pepsin activity and mucin content),–gastric mucosal levels of malondialdehyde (MDA) , tumor necrosis factor –alpha (TNF-alpha) , nitrite and prostaglandin E2 (PGE2) levels were also determined, in addition to the histopathologic examination of the gastric mucosa in each group.

Results: Nesfatin-1 displayed a significant ameliorative effect in gastric lesions induced by indomethacin as indicated by the histopathology and reduction in measured gastric juice parameters ($P<0.001$). It also reduced both gastric mucosal MDA and TNF-alpha significantly ($P< 0.001$) and increased nitrite and PGE2 levels in this ulcer model ($P< 0.001$).

Conclusion: Nesfatin-1 attenuates indomethacin induced gastric ulcer and potentiates ulcer healing in the stomach of rats exposed to chronic administration of indomethacin, this effect depend upon decrease in gastric secretion, increase in nitric oxide and PGE2, besides antioxidant and anti-inflammatory roles.

INTRODUCTION

Gastric ulcer is an illness that affects a considerable number of people worldwide. The etiological factors of this disorder include: stress, smoking, nutritional deficiencies, frequent and indiscriminate use of non steroidal anti-inflammatory drugs (NSAIDs) **(Khazaei and Salehi , 2006).**

The pathophysiology of gastric ulcer has generally focused on imbalance between aggressive and protective factors in the stomach (**Lima et al., 2006)**. The gastric ulcerogenic action of NSAIDs is believed to occur mainly due to their local inhibitory effect on gastric prostaglandin E2 (PGE2) and prostaglandin I2 (PGI2) that are the main inhibitors of gastric acid secretion (**Ribeiro-Rama et al., 2009).** In addition to oxidative gastric damage and the generation of pro-inflammatory mediators **(Kolgazi et al., 2014).**

Nesfatin-1 was recently identified as an anorexigenic factor associated with leptin and melanocortin signaling in the hypothalamus (**Darambazar et al., 2015).**This hypothalamic neuropeptide is composed of 82

amino acids with a molecular weight of 9.8 kDa, which is derived from the larger protein nucleobindin-2 (NUCB2**) (Oh-I et al., 2006).**

As a brain-gut peptide, nesfatin/NUCB2 is expressed in appetite-control hypothalamic nuclei, including the paraventricular nucleus of the hypothalamus (PVN), supraoptic nucleus (SON), arcuate nucleus (ARC), lateral hypothalamic area (LHA) (**Kohno et al., 2008)**. Additionally, peripheral tissues, such as the stomach, adipose tissue and pancreas were considered as sources for this peptide **(Stengel et al., 2009, 2013& Zhang et al., 2010& Foo et al., 2010& García-Galiano et al., 2010).**

Interestingly, NUCB2 mRNA expression is 10-times higher in the rat gastric mucosa than in the brain giving rise to a predominant production of nesfatin-1 in the stomach (**Stengel et al., 2009).** Nesfatin-1 was reported to regulate gastrointestinal motility and, in particular, it inhibits gastric contractions in the fasted state **(Watanabe et al., 2015)**. Whatever, the role of this peptide as related to gastric secretory function is still to be elucidated.

It is worth mentioning that nesfatin-1 has neuroprotective function in rats with subarachnoid hemorrhage through anti-inflammatory and antiapoptotic properties **(Ozsavci et al., 2011 & Tang et al., 2012)**. Accordingly, we hypothesized that, nesfatin-1 may play the same role regarding the gastric ulcer. So, this study aimed to investigate the possible ameliorative effect of nesfatin-1 against indomethacin induced gastric ulcer in rats, with exploration of the possible mechanisms underlying this effect.

ANIMALS AND METHODS

All the experimental procedures were conducted in accordance with the guiding principles for the care and use of research animals and were approved by the Institutional Review Board of Faculty of Medicine -Zagazig University.

Drugs and chemicals

Nesfatin-1 was obtained from Sigma chemical Co. (St. Louis, MO. USA) as a powder and was dissolved in normal saline. Indomethacin was obtained from Sigma chemical Co. (St. Louis, MO. USA) and was suspended in 0.5 % carboxymethylcellulose (Merck, Darmstadt, Germany).

Animals

24 adult male Albino rats weighing 190-210 grams were purchased from the animal house of Faculty of Veterinary Medicine-Zagazig University**.** Animals were given tap water ad libitum , fed with standard commercial rat chow and were left to accommodate for one week before dosing , kept 12 hours light ⁄ 12 hours dark regular cycle in partially humid and well aerated room.

Experimental design

The animals were randomly divided into 3 groups (8 rats per each): (I) Vehicle-treated control group: in which the animals received carboxymethylcellulose (0.5%) orally daily for 7 days then saline was administered intraperitoneally (i.p.) for 10 days – (II) indomethacin treated group (chronic gastric ulcer induction): the rats received oral indomethacin in a dose of 20 mg⁄ kg suspended in 0.5% of carboxymethylcellulose daily for 7 days (**Tariq et al., 2007),** then received i.p saline for 10 days. (III) indomethacin treated followed by nesfatin-1 : in which animals were given oral indomethacin in a dose of 20 mg⁄ kg suspended in 0.5% of carboxymethylcellulose daily for 7 days , then given Nesfatin-1 (1ug/Kg) intraperitoneal for 10 days **(Kolgazi et al., 2014)**.At 17th day , rats were fasted for 16 hours prior to sample collection **(Xia et al., 2012)**

Pyloric ligation

Pyloric ligation was carried out at the end of the study period in order to collect gastric secretion **(Alumets et al ., 1982)**. This was done under ether anesthesia , a midline abdominal incision was performed , the pyloric portion of the stomach was gently mobilized and carefully ligated with a silk ligature around the pyloric sphincter taking care not to interfere with gastric blood supply .Abdominal incision was sutured and the animals were allowed to recover from anesthesia ,the stomach was excised,

Analysis of gastric juice

Three hours later after the ligation, rats of all groups were euthanized with an overdose of ether, their stomachs were rapidly removed, opened by an incision along the greater curvature and the gastric juice were collected..

Gastric juice from each animal was centrifuged at 1000 g for 10 minutes to remove any solid debris and the volume of the supernatant was measured. The supernatant was then assayed for total acid concentration **(Hara et al., 1991)** , the total acid output was calculated by multiplying the volume of gastric juice by the total acid concentration, also pepsin activity **(Sanyal et al., 1971)** and mucin content **(Winzler, 1955)** were determined in the obtained Supernatant.

Biochemical analysis of gastric mucosa

The stomach was rinsed with saline and divided into 3 parts. Gastric mucosa of one part was scrapped and homogenized in 2 ml normal saline containing 0.1 M dithiothreitol and centrifuged at 2000 r.p.m for 10 minutes at room temperature. The supernatant was used for determination of prostaglandin E2 (PGE2) level by ELISA using PGE2 immunoassay kit (R&D systems, USA) according to **Granstrom et al. (1980).**

The mucosa of another part of the stomach was also scrapped, homogenized in cold potassium phosphate buffer (0.05 M, pH7.4) and centrifuged at 2000 r.p.m for10 minutes at 4^0C; the supernatant was then kept at – 80^0C for measurement of malonaldehyde (MDA) using Oxis kits (MDA586, Oxis International, Foster City, CA, USA) according to **Niehaus and Samuelsson, (1968)** and TNF-alpha using enzyme linked immunosorbent assay kit (Abcam, Catalog No. ab100785, USA) according to **He and Ting, (2002).** Also the total nitrate⁄nitrite in the gastric mucosal homogenate was assayed after reduction of nitrate to nitrite using the cadmium reduction method (**Sastry et al., 2002)**.

Histological studies

Histological studies were performed according to the method previously described **(Ogihara and Okabe , 1993)**. At autopsy, small pieces of tissue, including ulcers, were embedded in paraffin and sectioned at 5µm in an automated microtome. Haematoxylin and eosin staining was done. Tissue contraction, regeneration of the ulcerated mucosa, formation of granulation tissue, glands arrangement and inflammatory exudates were observed under the microscope.

Statistical analysis:

Data were presented as mean ± S.D Statistical significance was determined by one way analysis of variance (ANOVA) followed by LSD test, P values less than 0.05 were considered to be significant. In statistical analysis, SPSS version 18 program for Windows (SPSS Inc. Chicago, IL, USA) was used.

NB: To get the histopathological picture of the ulcer before healing a pilot study was done on the stomach mucosa on day seven of treatment with indomethacin.

RESULTS

Table (1) shows that indomethacin significantly increased gastric juice total acid output as compared to control group ($p<0.001$), in contrast Nesfatin-1 treatment significantly reduced total acid output ($p<0.001$) in group III. Pepsin activity was significantly increased by indomethacin ($p<0.001$) as compared with the control group. Rats treated with Nesfatin-1 showed significant ($p<0.001$) reduction in pepsin activity when compared with that of group II. Also, indomethacin significantly reduced gastric juice mucin content in group II compared to the control group . In nesfatin-1 treated rats, this value significantly ($p<0.001$) raised when compared with that of group II.

Table (1): Total acid output, pepsin activity and mucin content of gastric juice in all groups

Groups	**Total acid output (meq⁄3h)**	**Pepsin activity (µg⁄ml tyrosine)**	**Mucin content (mg%hexose)**
I. Control	49.4.4±3.03	119.5±4.3	85±3.2
II.Indomethacin treated	70.9±5.3 **a*** **	169.8±8.47 **a*** **	55.13±4.79 **a*** **
III.Indomethacin treated followed by nesfatin-1 treatment	60.5.±2.5 **a*** ,b****	130.48±5.43 **a*** ,b*** **	73.74±6.64 **a*** ,b*** **

***=significant (P<0.001), a (versus group I), b (versus group II)

Table (2) shows that indomethacin significantly increased gastric mucosal MDA levels as compared to control group ($p<0.001$), in contrast Nesfatin-1 treatment significantly reduced MDA **(**$p<0.001$) in group III. Gastric mucosal TNF-alpha levels was significantly increased by indomethacin ($p<0.001$) as compared with the control group. Rats treated with Nesfatin-1 showed significant ($p<0.001$) reduction in TNF-alpha levels when compared with that of group II. Also, indomethacin significantly ($p<0.001$) reduced both gastric mucosal content of PGE2 and nitrite in group II compared to the control group . In nesfatin-1 treated rats, those values significantly raised ($p<0.001$) when compared with that of group II.

Table (2): Gastric mucosal MDA,TNF-alpha,PGE2 and nitrite in all groups

Groups	**Malonaldhyde level (nmol/gwet Tissue)**	**TNF-alpha (pg/ml)**	**PGE2 (ngm/g wet Tissue)**	**Nitrite (nmol/gm wet Tissue)**
I. Control	19.1±0.2	290.5±12	319±10.5	165±3.59
II. Indomethacin treated	54.3±4.1 **a*** **	430±25 **a*** **	160±4.5 **a*** **	105.13±5.79 **a*** **
III. Indomethacin treated followed by nesfatin-1 treatment	26.6±2.2 **a***,b*** **	330±15 **a***,b*** **	210±±7.5 **a***,b*** **	129.8±6.64 **a***,b*** **

***=significant (P<0.001), a (versus group I), b (versus group II)

Histopathology of the stomachs

On 7 th day of indomethacin administration, treated rats in the pilot study showed sharply defined mucosal ulcer in stomach. Damaged mucosal epithelium, glands, inflammatory exudates, proliferated fibroblast and cellular debris was found in the ulcerated wall of stomach (Fig. 1b). No sign of healing was seen.

On 17th day of the study, group II rats showed reduced inflammatory exudates along with some extent of mucosal regeneration, glandular organization and reduced size of ulcer (Fig. 1c). Group III, showed minimal inflammatory exudates and proper organization of glands (Fig.1d).

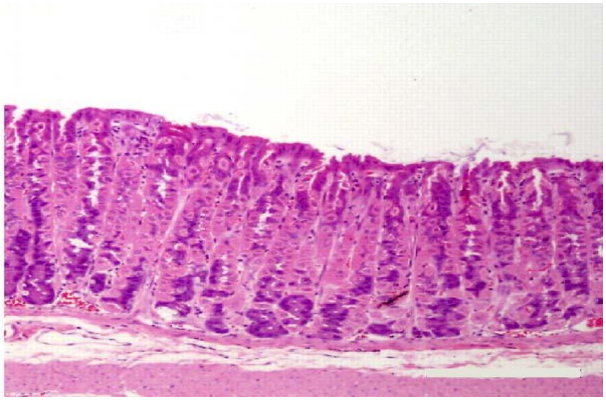

Fig. 1a: Gastric mucosa of control group (I)

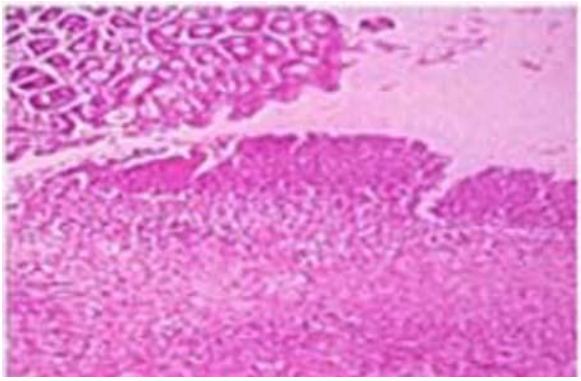

Fig .1b : Gastric mucosa On 7 th day of indomethacin administration

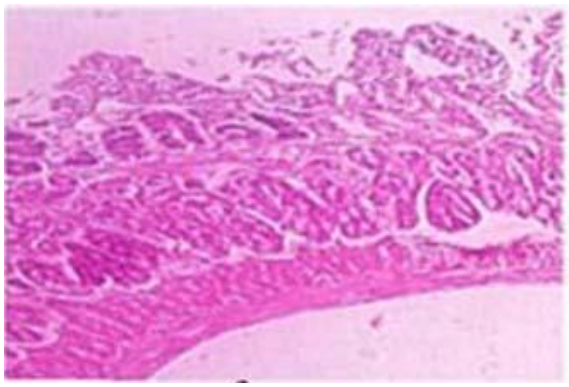

Fig.1c: Gastric mucosa on 17 th day of indomethacin administration in group II

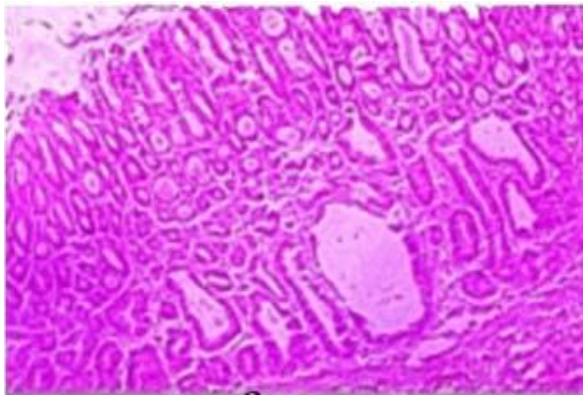

Fig .1d: Gastric mucosa On 17 th day of indomethacin administration in group III

Figure 1: Histopathological examination of the stomach mucosa.

DISCUSSION

The diversity of etiological factors underlying gastric ulcers and the complex nature of pathways participating in healing always make peptic ulcers treatment a complicated challenge **(El-Moselhy et al., 2009)**.

The current study highlights the alleviating effect of nesfatin-1, a newly identified anorexigenic peptide, on indomethacin induced gastric ulcer as it significantly improved all the deleterious effects induced by indomethacin on both gastric mucosa and gastric juice contents. This effect was associated with down regulation of inflammatory and oxidative stress markers together with increased in gastric mucosal levels of both PGE2 and nitric oxide.

Szlachcic et al. (2013), found that peripheral and central administration of nesfatin-1 inhibited stress induced increase in gastric acid and pepsin secretion in rats which is in keeping with the observations by **Xia et al. (2012)**, who also observed the inhibition of gastric secretion after a central administration of nesfatin-1 that involved the vagal mechanism.

In this regard, it is worthy to mention that, peripheral nesfatin-1 administrated by intravenous injection has been shown to be able to cross the blood-brain-barrier by a non-saturable mechanism **(Pan et al., 2007&Price et al., 2007).**

The result of the present work showed significant elevation in gastric MDA level in group II, an indicator for lipid peroxidation which is a well established mechanism for cellular injury **(Kwiecien et al., 2002)**. Nesfatin-1 treatment caused significant reduction in MDA level as compared to those of group II. It was reported that this peptide possesses free radicals scavenger activity (**Kolgazi et al., 2014)**. This suggests that nesfatin-1 accelerated ulcer healing via antioxidant activity.

The result of the current study showed significant elevations in gastric TNF-alpha in indomethacin treated group as compared to control group. This observation is in accordance with previous studies **(Zhang et al., 2008& Moustafa et al., 2013)**. Nesfatin-1 administration produced a significant reduction of TNF-alpha in the gastric mucosa, adding also anti-inflammatory role to its beneficial effects. This is in accordance with the results of other investigators (**Szlachcic et al., 2013&Kolgazi et al., 2014)**.

Nitric oxide (NO) plays a crucial role in the gastroprotection and ulcer healing mechanisms **(Laine et al., 2008).** The result of the present work showed marked reduction in mucosal nitrite level (as indicator of NO) in indomethacin treated group. This is attributed to the ability of indomethacin to upregulate the endothelin-1 leading to decrease production of gastric mucosal NO (**Slomiany and Slomiany, 2000**). Nesfatin-1 was able to increase the nitrite levels in this ulcer model in group III.

PGE2 influences virtually every component of the mucosal defense; stimulates mucus, bicarbonate; maintains mucosal blood flow; enhances the resistance of the epithelial cells to injury induced by cytokines **(Brzozowski et al., 2005**).In the present study , oral administration of indomethacin resulted in a significant reduction in gastric mucosal PGE2 level , which may be through inhibition of cyclooxygenase **(Schmassmann et al., 1998, .Bandyopadhyay et al., 1999)** . In our study rats treated with nesfatin-1 showed a significant increase in PGE2 level in gastric mucosa.

NO was reported to increase PGE2 biosynthesis in vivo through CGMP dependent mechanism **(Abi-Gerges et al., 2001)** and it is possible to assume that NO might regulate the release and or the biosynthesis of PGE2 in the stomach after damage during ulcer healing.

Our findings are supported by those of other researchers who reported that, gastroprotective effects of nesfatin-1 may involve endogenous PG and NO production, this notion is supported by observation that Nesfatin-1 exerts hyperemic effect on the gastric mucosa and that the selective or non-selective cyclooxygenase (COX) blockers reduced this effect (**Szlachcic et al., 2013).** Furthermore, they reported that the protective and hyperemic effects of nesfatin-1 were lost in rats with capsaicin-denervation and those pretreated with capsazepine, the antagonist of vanilloid (TRVP1) receptors, which supports the notion that this peptide may trigger the sensory afferent endings to release vasodilatory and protective neuropeptides such as calcitonin gene related peptide (CGRP). These observations suggest that CGRP and vanilloid receptors can cooperate with PG and NO in the mechanism of nesfatin-1-induced gastroprotection.

In controversy, **Angelone et al. (2013)** concluded that Nesfatin-1 cannot produce any significant change in NO levels in the heart in vitro.

Conclusion: Nesfatin-1 attenuates indomethacin induced gastric ulcer and potentiates ulcer healing in the stomach of rats exposed to chronic administration of indomethacin and this effect depend upon decrease in gastric secretion, increase in nitric oxide and prostaglandin E2, besides an antioxidant anti-inflammatory role.

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