Gastroprotective Effect of Vanillin on Indomethacin-Induced Gastric Ulcer in Rats: Protective Pathways and Anti-Secretory Mechanism

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Abstract

Indomethacin provokes aggressive ulcerogenic adverse effects. Natural products with fewer side effects are therefore highly requested to attenuate its gastric ulcer effect. Vanillin is a natural compound widely used as a flavoring agent which has antioxidant activity. Therefore, the aim of this study was to investigate its gastroprotective effect against indomethacin induced gastric injury. Rats were divided into four groups; first group served as control, group 2: Treated with indomethacin (25 mg/kg, po.), group 3: Pre-treated with ranitidine (reference drug) (50 mg/kg, po., 5 days) before indomethacin and group 4: Pretreated with vanillin (100 mg/kg, po., 5 days). Pre-treatment with vanillin reduced ulcer index, gastric juice volume, free, total acidity and histopathological changes induced by indomethacin. Although it reduced gastric oxidative stress, it elevated enzymatic antioxidant activity and gastric nitric oxide content. Moreover, it reduced gastric NFκB protein expression and activity as well as inhibition in levels of pro-inflammatory cytokines, Myeloperoxidase (MPO) and caspase 3 activities. It down regulated gene expression of TNF-α, Cytokine-Induced Neutrophil Chemo Attractant (CINC-2α) and caspase-9 while lacking effect on mucosal prostaglandin E2 (PGE2) level. Collectively, vanillin displayed gastroprotective effects in indomethacin induced gastric ulcer by anti-secretory action and cytoprotective effect via antioxidant and anti-inflammatory activities.

Keywords: Vanillin; Indomethacin; Gastric ulcer; NFκB expression; TNF-α; CINC-2α; Caspase-9

Introduction

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are extensively used for their analgesic, anti-inflammatory and antipyretic properties. However, their administration is frequently associated with peptic ulcer induction [1]. Indomethacin is an important member of the NSAIDs family. It is still extensively used in joint stiffness, arthritis, and obstetrics to delay uterine contractions. In addition it is used in the neonatal unit to facilitate patent ductus arteriosus closure. However it provokes aggressive ulcerogenic potential in both animals and humans [2]. Many previous studies tried to decrease damaging indomethacin effect on gastrointestinal mucosa [3-5].

Indomethacin induces its gastrointestinal toxicity via several mechanisms such as an increase in gastric acid secretion; interfere with mucosal cell regeneration via inhibition of PGE2 synthesis, production of free radicals, reduction of gastric nitric oxide level and invasion of activated neutrophils as well as induction of gastric cells apoptosis [6].

Vanillin (4-hydroxy-3-methoxybenzaldehyde) originally isolated from Vanilla planifolia. It is commonly used as a flavoring agent. However, it has many beneficial biochemical and pharmacological activities. Previous studies showed that vanillin has antioxidant, anti-inflammatory, inhibition mutagenesis and anti-carcinogenic effect [7].

Although many lines of substantiation supporting the beneficial effect of vanillin, its gastroprotective effect against indomethacin-induced gastric ulcer has not been yet thoroughly investigated. Therefore, the present study was conducted to evaluate the therapeutic potential of vanillin in peptic ulcer induced by indomethacin using ranitidine as a reference standard drug.

Material and Methods

Animals

Adult male Wistar albino rats weighing 150-200 g were placed in polyethylene cages in groups of 6. The experiment was conducted under controlled laboratory conditioning where food and water were provided ad libitum. This study was carried out in accordance to the guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press) and the experimental protocol approved by the institutional research ethics committee at Faculty of pharmacy, Damanhour University.

Drugs and chemicals

Indomethacin (Sigma Chemical Co., MO, and USA) was suspended in 1% tween 80 however vanillin and ranitidine hydrochloride (Sigma Chemical Co., MO, USA) dissolved in distilled water. All other chemicals used were of good quality and analytical grade.

Experimental design

Rats were randomly divided into four experimental groups, each consisting of six animals (n=6); first group served as normal control received vehicles orally by intragastrical gavage, second group administered indomethacin (25 mg/kg, intragastrically) [8], third group: Rats received ranitidine (used as a standard reference drug) orally for 5 days (50 mg/kg) [4] before indomethacin administration.
and group 4: Treated with vanillin orally (100 mg/kg) for 5 days before indomethacin treatment. All animals were fasted 24 h. prior to indomethacin administration except for water to prohibit exogenous dietary effect.

Rats in the first set were anesthetized 4 h. after indomethacin administration and the abdomen were opened with clamping of pylorus to collect gastric juice then their stomach were removed, opened along the greater curvature, and washed with cold saline. Gastric juices were collected and centrifuged for 5 mints at 2000 × g and collected supernatants. Also, the extent of gastric lesions (ulcer index, UI) was calculated by the formula: UI=10/(total mucosal area/total ulcerated area) [9] then the stomach of each rat were cut into multiple pieces for assessment of gastric mucosal damage and histopathological examination.

Determination of free, total acidity and pH of gastric juice

Gastric juice was collected, centrifuged and the supernatant was titrated with 0.01 N NaOH using methyl orange as an indicator until yellowish orange color come out and the result indicated free acidity. Then phenolphthalein was added as an indicator and continues titrating until red color reappears however the total volume of alkali added indicated total acidity as we described before [4]. Moreover, pH of gastric juice was determined by digital pH meter.

Determination of gastric oxidative stress and enzymatic antioxidant activity

Gastric levels of Thiobarbituric Acid Reactive Species (TBARs) and reduced Glutathione (GSH) using a commercially available ELISA kits according to manufacturer's instructions (Cayman, Ann Arbor, MI). Also, gastric levels of Superoxide Dismutase (SOD) and Catalase (CAT) were measured according to manufacturer's instructions (Cayman, Ann Arbor, MI) as markers of enzymatic antioxidant activity.

Determination of gastric tissue levels of inflammatory markers

Gastric tissues were homogenized with 50mM phosphate buffer (pH 7.4) then centrifuged at 11,000 × g for 20 mints at 4°C then the supernatants were utilized for assessment of levels of inflammatory cytokines TNF-α ELISA Kit (ab100785, abcam, Cambridge, MA, USA), IL-1β (ab100768), and IL-6 (ab100772) IL-10 (ab100765). Moreover, gastric caspase-3 activity was determined by caspase-3 ELISA kit according to manufacturer’s instructions (Cayman, Ann Arbor, MI) as markers of enzymatic antioxidant activity.

Determination of gastric tissue level of NFκB activity

Nuclear extract was prepared by a nuclear extract kit (Cayman, Ann Arbor, MI) from the gastric tissue. The nuclear extract was used for the assessment of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activity using NFκB p65 transcription factor assay kit (Cayman, Ann Arbor, MI) then optical density was normalized per ng of nuclear extract protein. Data was expressed as relative to mean of control group X100.
12,000 × g for 10 mints and the level of prostaglandin E2 (PGE2) were evaluated using a commercially available ELISA kits according to manufacturer's instructions (Cayman, Ann Arbor, MI).

**Determination of mucosal hexosamine content**

Hexosamine mucous content is an index of mucous biosynthesis was determined by extraction of gastric mucosal mucin of the fourth set using Triton X-100 then hydrolyzed by HCL 4 N. Neuhaus and Letzring method was applied to evaluate hexosamine using glucosamine (Sigma Chemical Co., MO, USA) as a standard. As described before [12] the standard and the prepared samples were incubated with acethylacetone at 100ºC for 15 mints then mixed with Ehrlich's reagent for 40 mints and the concentration of gastric mucosal hexosamine was measured spectrophotometrically at 550 nm.

**Histopathological evaluation**

Gastric tissue samples were fixed in buffered 10% formalin and processed for histopathological examination as described before [13]. Briefly, four micrometer-thick paraffin sections were prepared and stained with haematoxylin and eosin for light microscope examination (magnification X20) by double blind pathologist.

**Statistical analysis**

All data are presented as mean ± standard error and analyzed with one-way ANOVA followed by Tukey's post hoc test for multiple group comparisons. Analyses were carried out using GraphPad Prism Version 4.0 software (GraphPad Software Inc., La Jolla, CA). For all comparisons, P<0.05 was regarded as statistically significant.

**Results**

**Antiulcer characteristics of vanillin**

Treatment with indomethacin produced significant increase in ulcer index and ulcer score while pre-treatment with vanillin or ranitidine reduced areas and severity of gastric lesions in the term of ulcer score and index vs. indomethacin treated animals (Table 1). Also, histopathological findings confirmed the ability of vanillin to improve indomethacin-induce gastric ulcer in the gastric mucosa compared to the normal control group. The indomethacin-administrated rats showed extensive gastric lesion ulceration with inflammatory cells infiltration and edema in submucosal layer however, sections from rats pre-treated with ranitidine showed markedly reduced gastric lesions with edema and absence of inflammatory cells infiltration in submucosal layer compared to indomethacin-treated animals (Figure 1).

**Effect of vanillin on gastric secretion and acidity**

Treatment of rats with indomethacin caused significant increase in volume of gastric juice as well as free and total acidity of gastric secretion vs. control group however pre-treatment with vanillin or ranitidine produced decrease in volume, free and total acidity of gastric secretion compared to indomethacin-treated animals. Moreover, there was no significant difference between vanillin and ranitidine treated groups (Table 1).

**Effect of vanillin on gastric tissue levels of lipid peroxides**

Oral administration of indomethacin induced significant increase in gastric level of TBARs while it decreased gastric GSH level, SOD and CAT activities compared to control group. Although pretreatment with ranitidine or vanillin produced decrease in gastric level of TBARs, it...
increased GSH level, SOD and CAT activities vs. indomethacin-treated animals. Moreover, rat pretreated with vanillin significant decreased gastric TBARs level whereas increased level, SOD and CAT activities compared to Ran-treated animals (Figure 2).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>INDO</th>
<th>Ran+INDO</th>
<th>Van+INDO</th>
</tr>
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<tbody>
<tr>
<td>Ulcer index</td>
<td>0</td>
<td>0.61 ± 0.04*</td>
<td>0.16 ± 0.05$</td>
<td>0.18 ± 0.06$</td>
</tr>
<tr>
<td>Ulcer score</td>
<td>0</td>
<td>3.16 ± 0.30*</td>
<td>1.00 ± 0.35$</td>
<td>1.16 ± 0.39$</td>
</tr>
<tr>
<td>Gastric juice pH</td>
<td>3.26 ± 0.07</td>
<td>2.21 ± 0.08*</td>
<td>3.03 ± 0.16$</td>
<td>2.86 ± 0.05$</td>
</tr>
<tr>
<td>Gastric volume (ml)</td>
<td>1.86 ± 0.19</td>
<td>4.25 ± 0.28*</td>
<td>2.00 ± 0.21$</td>
<td>2.60 ± 0.35$</td>
</tr>
<tr>
<td>Free acidity (Meq/l)</td>
<td>31.93 ± 3.81</td>
<td>78.56 ± 2.25*</td>
<td>34.49 ± 4.12$</td>
<td>44.96 ± 4.40$</td>
</tr>
<tr>
<td>Total acidity (Meq/l)</td>
<td>42.57 ± 4.29</td>
<td>97.09 ± 3.43*</td>
<td>59.20 ± 5.32$</td>
<td>59.72 ± 5.53$</td>
</tr>
</tbody>
</table>

Table 1: Effect of vanillin (Van) or ranitidine (Ran) on ulcer index, ulcer score, gastric juice free acidity and total acidity in indomethacin (INDO)-induced gastric ulcer. Data are expressed as mean ± standard error (n=6; *P<0.05 versus control group, $P<0.05 versus INDO group and #P<0.05 versus Ran+INDO group).

Effect of vanillin on gastric NFκB-p65 expression and NFκB-p65 activity

In Indomethacin group; NFκB-p65 expression and NFκB-p65 activity were significantly higher than in normal control while pre-treatment with vanillin or ranitidine caused significant decrease in NFκB-p65 expression and NFκB-p65 activity compared to indomethacin-treated group. Moreover, there was no significant difference between vanillin and ranitidine groups (Figure 3).

Effect of vanillin on gastric tissue levels of inflammatory cytokines

Oral treatment with indomethacin induced significant increase in gastric levels of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 while it decreased anti-inflammatory cytokines IL-10. However treated rats with vanillin or ranitidine decreased gastric levels of TNF-α, IL-1β, IL-6 even as it increased gastric level of IL-10 compared to indomethacin-treated animals. Pre-treatment with vanillin caused significant decrease in gastric level of TNF-α and IL-1β and there was a significant increase in gastric level of IL-10 vs. ranitidine-treated animals while there was no significant difference in gastric level of IL-6 between vanillin and ranitidine treated groups (Figure 4). In addition, there was significant down regulation of gastric gene expression of TNF-α and CINC-2α of vanillin or ranitidine group vs. indomethacin group.

Effect of vanillin on gastric tissue of MPO activity and apoptosis

In indomethacin group; MPO and caspase 3 activities were significantly increased vs. normal control group while vanillin or ranitidine decreased MPO and caspase 3 activities vs. indomethacin group. In addition, vanillin decreased MPO and caspase 3 activities vs. ranitidine group (Figure 5).
Effect of vanillin on gastric mucosal barrier, mucosal levels of hexosamine and PGE2

Oral administration of indomethacin caused significant decrease in gastric mucosal barrier, mucosal levels of hexosamine (index of mucosal synthesis) and PGE2 vs. control group. Although pretreatment with vanillin increased mucosal barrier and synthesis, it failed to change mucosal level of PGE2 vs. indomethacin group (Figure 7).

Discussion

Non-steroidal anti-inflammatory drugs such as indomethacin are extensively recommended in the management of pain, fever and inflammation while its GIT adverse effects disturb its use [13]. There are a variety of pathogenic mechanisms that may contribute to the formation of a peptic ulcer via unbalance between aggressive factors and decreased gastric resistance [6].

Present study revealed that indomethacin induced aggressive factors by via increase gastric juice volume, free and total acidity as well as a decrease in gastric juice pH indicated altered hydrophobicity. Similarly, Sabiu et al. [14] and his colleagues proved stimulation of gastric acid secretion by indomethacin [14]. Pre-treatment with vanillin has an ability to decrease gastric acid secretion induced by indomethacin. Gastric secretion is a controlled by multiple mechanisms; neural, paracrine, and hormonal pathways. Histamine increase acid secretion through its binding to H2 receptors [15]. Vanillin's inhibitory action on histamine release may be playing a role in its effect on acid secretions [16]. Also, vanillin produced a reduction in the histamine secreting mast cells in ethanol-induced peptic ulcer by reduction of mast cell degranulation [17]. Moreover, it was proved that vanillic acid, a metabolite oxidized product of vanillin, suppresses the mast cell-mediated allergic inflammatory response by blocking the signaling pathways downstream of high affinity IgE receptor on the surface of...
mast cells [18]. Therefore, the gastroprotective effect of vanillin may be ascribed to the reduction of mast cell degranulation.

Free radicals play a critical part in pathophysiology mechanism of NSAIDs-induced peptic ulcer [19]. The present study revealed induction of gastric level of TBARs by oral treatment with indomethacin however these free radicals depleted GSH gastric content. Also, indomethacin reduced SOD and CAT activities; these enzymes reduce intracellular levels of superoxide radicals. SOD enzyme renovates superoxide anion into H$_2$O$_2$, which then decomposes into water via CAT and Glutathione Peroxidase (GSHPx) [8]. Indomethacin inhibited the mitochondrial oxidative phosphorylation leading to the release of cytochrome c from mitochondrial inter membranous space into cytosol and to the release of ROS such as superoxide anion and H$_2$O$_2$. These free radicals declined the intracellular ATP concentration, leakage of Ca$^{2+}$ out of mitochondria, cellular osmotic imbalance and lipid peroxidation, resulting in increased permeability and subsequent mucosal damages [6]. Meanwhile, pre-treatment with vanillin in this study reduced gastric levels of TBARs and increased GSH level, SOD and CAT activities indicating a decrement in the indomethacin induced oxidative stress and this was superior to ranitidine pre-treatment. These findings are in harmony with earlier reports who noted the antioxidant property of vanillin [20-23].

Indomethacin encourages direct adherence of neutrophils to the gastric endothelium which occluding micro vessels with subsequent decrease in mucosal blood flow leading to ulceration [24]. MPO is the main marker of neutrophil infiltration which catalyzes the production of toxic hypochlorous acid from H$_2$O$_2$ as well as oxidation of tyrosine to tyrosyl radical using H$_2$O$_2$ as an oxidizing agent then causing inflammation and induction of apoptosis [25]. The current study showed the inhibitory action of vanillin on MPO induced by indomethacin in part due to oxidative scavenging property of vanillin on H$_2$O$_2$. Also, pre-treatment with vanillin inhibited MPO activity in peptic ulcer induced by ethanol [17].

NF-κB is a transcription multiprotein complex that can activate inflammatory cytokynes genes therefore its pathway is considered a key player in the inflammation [26,27]. NF-κB consists mainly of p50 and p65 subunits however the p65 subunit is responsible for the strong transcription activating potential of NF-κB [28]. Our results displayed stimulation of NF-κB expression and NF-κB p65 activity in gastric tissue by treatment with indomethacin. Since gastric ulcers are associated with inflammation, indomethacin activates NF-κB subsequently NF-κB translocates into the nucleus to up regulate the expression of pro-inflammatory cytokynes genes such as TNF-α and cytokine-induced neutrophil chemo attractant (CINC-2α). These data were confirmed by induction of gastric tissue levels of pro-inflammatory cytokynes such TNF-α, IL-1β and IL-6 as well as decreasing gastric level of anti-inflammatory cytokine IL-10 by indomethacin treatment. Also, Takahashi et al. [29] noted the amplifying in NF-κB and the up regulation of the expression of healing-promoting factors; cyclooxygenase-2 (COX-2), inducible NOS, CINC, and IL-1β [29]. Antonisamy et al. [30] proved that indomethacin increased IL-6, IL-1β, TNF-α, interferon-γ (IFN-γ) levels with decreased of anti-inflammatory cytokynes IL-10 and IL-4 [30].

Similar to ranitidine, pre-treatment with vanillin resulted in the down regulation of the indomethacin induced decrease in NF-κB expression and NF-κB p65 activity resulting in a decrement in tissue gastric levels of inflammatory cytokynes TNF-α, IL-1β and IL-6 in addition to an increase in anti-inflammatory cytokine IL-10 indicating anti-inflammatory action of vanillin via embarrasment of NF-κB pathway. Previous studies also noted vanillin's anti-inflammatory property [16-32]. Moreover it was proved that also vanillic acid regulated ulcerative colitis via inhibition NF-κB p65 activation and reduction in pro-inflammatory cytokynes production; IL-1, IL-6 and TNF-α [33].

Oxidative stress and inflammation is a major inductor of cell death by apoptosis through intrinsic pathway caspase 9 then activation of caspase 3 [4]. indomethacin induced apoptosis via up regulation of caspase 9 gene expression leading to increase caspase 3 activity while pre-treatment with vanillin attenuated these alterations may be as a result of antioxidant and anti-inflammatory actions.

Nitric oxide (NO) is a mediator of gastrointestinal mucosal defense but inconsistently, it also contributes to mucosal damage. Although NOS is responsible for synthesis of nitric oxide is has many isoforms. Mainly, cytoprotective endothelial (eNOS) and cytotoxic inducible (iNOS) [13]. Nitric oxide from eNOS improves the mucosal blood flow, protects the integrity of epithelial tissue and inhibits activation, adhesion and migration of leucocytes in the inflammatory [34] resulting in increasing mucus synthesis and accelerating ulcer healing [12]. This study showed down regulation of treatment with indomethacin to gastric tissue eNOS gene resulting in a decrement in gastric level of NO leading to a decrease in mucosal synthesis and mucosal barrier content which confirmed by biochemical and histopathological analysis. Similarly, previous studies showed a decrease in NO production by indomethacin [34-37]. Meanwhile pre-treatment with vanillin increased eNOS gene expression as well as gastric level of NO leading to increasing mucus synthesis plus restoration of the depleted gastric mucus levels. Kumar et al. [38] showed up regulation action of vanillic acid on eNOS in NO deficient rat.

It has been reported that PGE2 prevents gastric mucosal damage by indomethacin in humans and animals [39,40] however this study indicated that pre-treatment with vanillin had no effect on the gastric mucosal PGE2 concentration. On contrary, our reference drug (ranitidine) showed an increase in mucosal PGE2 level. Therefore, the inhibition of mucosal lesions by vanillin cannot be attributed to PG synthesis.

In conclusion, oral treatment with vanillin displayed significant gastroprotective effects in indomethacin induced gastric ulcer by anti-inflammatory action and cytoprotective effect via anti-oxidant and anti-inflammatory activities as well as modulation of nitric oxide gastric tissue content lacking the effect on prostaglandins. Vanillin might have the potential for further development as a promising drug for anti-ulcer treatment. Accordingly, the current study indicated vanillin in a clinical setup to be a promising natural drug in pre-and concurrent treatment with NSAIDs administration to decrease their gastro toxic effects. Vanillin might have the potential for further development as a promising drug for anti-ulcer treatment.

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damage and content of prostacyclin and prostaglandin E2. Prostaglandins 30: 609-618.