Wheat peptides reduce oxidative stress and inhibit NO production through modulating μ-opioid receptor in a rat NSAID-induced stomach damage model

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[ABSTRACT] Non-steroidal anti-inflammatory drugs (NSAIDs) induce tissue damage and oxidative stress in animal models of stomach damage. In the present study, the protective effects of wheat peptides were evaluated in a NSAID-induced stomach damage model in rats. Different doses of wheat peptides or distilled water were administered daily by gavage for 30 days before the rat stomach damage model was established by administration of NSAIDs (aspirin and indomethacin) into the digestive tract twice. The treatment of wheat peptides decreased the NSAID-induced gastric epithelial cell degeneration and oxidative stress and NO levels in the rats. Wheat peptides significantly increased the superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities and decreased iNOS activity in stomach. The mRNA expression level of μ-opioid receptor was significantly decreased in wheat peptides-treated rats than that in the control rats. The results suggest that NSAID drugs induced stomach damage in rats, which can be prevented by wheat peptides. The mechanisms for the protective effects were most likely through reducing NSAID-induced oxidative stress.

(KEY WORDS) Wheat peptides; Stomach damage; Oxidative stress; iNOS; μ-Opioid receptor


Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly prescribed drugs for arthritis, inflammation, and cardiovascular protection. However, these drugs often cause gastrointestinal complications [1-2]. The pathophysiology of these complications had mostly been ascribed to NSAID’s inhibitory effects on cyclooxygenases [3], increased oxidative stress, and subsequent prostaglandin deficiency [4-5]. Recent clinical results have demonstrated that the prevalence of NSAIDs-induced stomach damage is more often than previously expected [6]. The use of NSAIDs is recently increasing; therefore, increased awareness of their gastrointestinal side effects is needed. However, effective prevention and treatment of the side effects of NSAIDs in stomach have not yet been developed.

Wheat peptides contain substances with biological functions beyond basic nutrition, and extensive research has been undertaken to identify and characterize these biologically active peptides [7-8]. There are diverse biological functions attributed to wheat peptides, including antioxidant [9], antihypertensive [10], antimicrobial [11] and anticancer activities [12].

Our previous work has suggested that wheat protein-derived bioactive peptides can exert growth promotion effects on rat stomach epithelial cells. Our previous results also indicate that wheat peptides exhibit protective effects against small intestinal damage induced by NSAID drugs in rats. However, little work has been done to investigate the protective effects of wheat peptides on stomach damage and underlying mechanisms of action.

Materials and Methods

Chemicals and reagents

Wheat peptides were obtained from China National Re-
search Institute of Food & Fermentation Industries (Beijing, China). The molecular weights of the wheat peptides were 140–1 000 Da, which were measured using the protease hydrolysis method.

The assay kits for analyses of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), inducible nitric oxide synthase (iNOS), NO, and malondialdehyde (MDA) were purchased from Jiancheng Biologic Project Company (Nanjing, China). The TRIZOL Reagent Kit was purchased from Tian Gen Biotech Co. (Beijing, China). M-KGV reverse transcriptase, RNase inhibitor, and dNTP mixtures were obtained from Promega (Madison, USA). The Ex Taq DNA polymerase was from TaKaRa Bio Inc. (Shiga, Japan). All the other chemicals and reagents were of analytical grade.

**Wheat peptide components**

Table 1 shows the composition of the wheat peptides. Wheat peptides from 140 to 1 000 Da counted for 92% of the total prepared wheat peptides and Wheat included 3–6 amino-acid residues.

<table>
<thead>
<tr>
<th>Item</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>98.3 ± 0.56</td>
</tr>
<tr>
<td>Lipid</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Ash content</td>
<td>4.56 ± 0.25</td>
</tr>
<tr>
<td>Water</td>
<td>4.21 ± 0.11</td>
</tr>
</tbody>
</table>

**Animals**

Fifty male Sprague-Dawley (SD) rats (weighing 200–250 g) were used for this study. They were housed under controlled environmental conditions of temperature (22 ± 2 °C) and a 12 h/12 h light/dark cycle, and maintained on (unless otherwise stated) standard food pellets and tap water. All animal handling procedures were performed in accordance with the ‘Principles of laboratory animal care’ of NIH and the protocol for animal study of Animal Management Committee of Jiangsu Province and Southeast University.

**Experimental design**

The rats (n = 50) were randomly divided into five groups:

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
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<tbody>
<tr>
<td>I</td>
<td>10</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
</tr>
<tr>
<td>III</td>
<td>10</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
</tr>
<tr>
<td>V</td>
<td>10</td>
</tr>
</tbody>
</table>

Group I (n = 10): normal control rats with free access to normal diet for 30 days.

Group II (n = 10): model control rats with free access to a normal diet for 30 days.

Group III (L, n = 10): wheat peptide treated rats; the animals were put on a normal diet and treated with the low dose of wheat peptide (20 mg·kg⁻¹·d⁻¹, intragastric administration) for 30 days.

Group IV (M, n = 10): wheat peptide treated rats, the animals were put on a normal diet and treated with the moderate dose wheat peptide (100 mg·kg⁻¹·d⁻¹, intragastric administration) for 30 days.

Group V (H, n = 10): wheat peptide treated rats, the animals were put on a normal diet and treated with the high dose wheat peptide (500 mg·kg⁻¹·d⁻¹, intragastric administration) for 30 days.

On the last day of experiment, the rats from group II, III, IV and V were given aspirin (300 mg·kg⁻¹) and indomethacin (50 mg·kg⁻¹) by intragastric administration. The same treatment was repeated 45 min later. At 2 h after the second dosing, the animals were sacrificed by cervical dislocation and the stomachs were collected. A part of the stomach was fixed in 10% neutral buffered formalin and the rest was rapidly frozen in liquid nitrogen and stored at −80 °C until analysis.

**Analytical methods**

**Histologic observation**

Tissue specimens were fixed in 10% neutral buffered formalin for 24 h, then dehydrated in increasing concentrations of ethanol and xylene, and embedded in paraffin. Five-μm thick sections were cut and placed onto glass slides coated with 10% poly-L-lysine. The slides with sections were incubated at 50 °C for 45 min to improve the adherence of tissue sections to the glass, followed by staining with hematoxylin and eosin. Histological changes were observed by light microscopic examination (Olympus BH2, Tokyo, Japan) at a magnification of 200 ×. Briefly, four sections of stomach tissues from each rat were examined and quantified under a microscope, and ten fields were selected randomly per section. Evaluation of damage was semi-quantified using a score of 1, 2, or 3 according to the number of gastric epithelial cell degeneration and some inflammatory cell infiltration in a microscopic field where 1 = no or few gastric epithelial cell degeneration present, 2 = moderate gastric epithelial cell degeneration, and 3 = marked gastric epithelial cell degeneration and some inflammatory cell infiltration. Evaluation of each section was carried out in a blinded fashion by two independent pathologists.

**Assays of the MDA, NO level, iNOS and antioxidant activity in stomach tissues**

The 10% homogenates of stomach were prepared in the phosphate buffer (0.1 mol·L⁻¹, pH 7.4) containing 1 mmol ethylenediaminetetra acetic acid (EDTA), 0.25 mmol·L⁻¹ sucrose, 10 mmol·L⁻¹ potassium chloride (KCl), and 1 mmol·L⁻¹ phenylmethyl sulfonyl fluoride (PMSF).

Lipid peroxidation was determined by quantifying MDA concentrations, which was spectrophotometrically measured by the absorbance of a red-colored product with thiobarbituric acid [13-14].

The Nitric oxide (NO) release was determined spectro photometrically by measuring accumulation of nitrates, as described previously [15]. The absorbance was read at 540 nm and sodium nitrite was used as standard.

SOD, GPx, CAT and iNOS activities were determined using respective kits. Briefly, the determination of SOD activity was based on the production of O²⁻ anions by the xanthine/xanthine oxidase system [16]. The amount of SOD that inhibited 50% the rate of reduction under the specified conditions was regarded as one enzyme unit.
The reduced glutathione (GSH) was catalyzed by glutathione peroxidase (GPx) in the presence of hydrogen peroxide. GPx activity was estimated by the analysis of GSH in the enzymatic reaction. One unit of enzyme activity represents a decrease in GSH concentration of 1 μmol·L⁻¹ per minute after subtraction of non-enzymic mode.

The catalase activities were determined as described by Sozmen, in which the degradation of H₂O₂ was recorded. The results were expressed as mean ± SD of 10 rats per group and the statistical significance was evaluated by one-way analysis of variance (ANOVA), followed by the Dunnett's post hoc test, using the SPSS/15.0 software. Significantly less gastric epithelial cell degeneration and tissue damage were observed in the rats treated with the low and moderate dose of wheat peptides, compared with the model control group (Figs. 1C and 1D). The high dose group showed no sign of stomach damage compared with the model control group (Figs. 1C and 1D).

Histological analysis

No obvious histologic damage was observed in the normal control group (Fig. 1A). As shown in Fig. 1B, NSAIDs induced gastric epithelial cell degeneration (aplanate shape) and inflammatory cell infiltration in stomach in the rats. Significantly less gastric epithelial cell degeneration and tissue damage were observed in the rats treated with the low and moderate dose of wheat peptides, compared with the model control group (Figs. 1C and 1D). The high dose group showed no sign of stomach damage (Fig 1E). Semi-quantification of histologic evaluation was presented in Table 4.

**Table 3** Primer pairs and PCR conditions for analyses of µ-opioid receptor, NOS, and β-actin genes

<table>
<thead>
<tr>
<th>gene</th>
<th>Primers sequence(5’–3’)</th>
<th>Orientation</th>
<th>PCR conditions</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>atcgctgcgtgacattaaagaga</td>
<td>Forward</td>
<td>95 °C 5 min, 33 cycles (95°C, 30 s, 60 °C, 40 s, 72 °C, 60 s), 72 °C 10 min, 4 °C</td>
<td>429</td>
</tr>
<tr>
<td></td>
<td>ggacatgtgggagcattagag</td>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>µ opioid receptor</td>
<td>tgccttcctcaggtctcaact</td>
<td>Forward</td>
<td>95 °C 5 min, 33 cycles (95°C, 30 s, 60 °C, 40 s, 72 °C, 60 s), 72 °C 10 min, 4 °C</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>aacatcagggcagccactgag</td>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>acaggagacagcctaatcaag</td>
<td>Forward</td>
<td>95 °C 5 min, 33 cycles (95°C, 30 s, 55 °C, 40 s, 72 °C, 60 s), 72 °C 10 min, 4 °C</td>
<td>390</td>
</tr>
<tr>
<td></td>
<td>cttcgaggtcagatagag</td>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Statistical analysis**

The results were expressed as mean ± SD of 10 rats per group and the statistical significance was evaluated by one-way analysis of variance (ANOVA), followed by the Dunnett's post hoc test, using the SPSS/15.0 software. P < 0.05 was considered statistically significant.

**Results**

**Histological analysis**

No obvious histologic damage was observed in the normal control group (Fig. 1A). As shown in Fig. 1B, NSAIDs induced gastric epithelial cell degeneration (aplanate shape) and inflammatory cell infiltration in stomach in the rats. Significantly less gastric epithelial cell degeneration and tissue damage were observed in the rats treated with the low and moderate dose of wheat peptides, compared with the model control group (Figs. 1C and 1D). The high dose group showed no sign of stomach damage (Fig 1E). Semi-quantification of histologic evaluation was presented in Table 4.

**Change of MDA level and antioxidase activity of stomach**

As shown in Fig. 2, there was a significant increase in the MDA level in stomach obtained from the model control animals when compared with the normal control animals (P < 0.05).
Fig. 1  Histologic observation of stomach
A: Control, B: Model Control, C: Low dose of wheat peptides treated, D: moderate dose of wheat peptides treated, E: High dose of wheat peptides treated

Table 4  Histological evaluation of stomach tissue (mean ± SD, n = 10)

<table>
<thead>
<tr>
<th>Histologic evaluation</th>
<th>Control</th>
<th>Model Control</th>
<th>Low dose</th>
<th>Moderate dose</th>
<th>High dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2.21 ± 0.06*</td>
<td>1.48 ± 0.03*</td>
<td>1.53 ± 0.04*</td>
<td>2.12 ± 0.10</td>
</tr>
</tbody>
</table>

*P < 0.05 vs the control group; #P < 0.05 vs the model control group

The treatments with the low and moderate doses of wheat peptides significantly reduced the MDA level, compared with the model controls (P < 0.05).

As shown in Table 5, there was a significant increase in the activities of SOD and GPx in the model control rats, compared with the normal controls (P < 0.05). The low and moderate doses of wheat peptides significantly increased the activities of SOD and GPx in stomach compared to the model control rats (P < 0.05).

Table 5  Change of antioxidase activity of stomach (mean ± SD, n = 10)

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (U/mg pro)</th>
<th>CAT (U/mg pro)</th>
<th>GPx (U/mg pro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>70 ± 6</td>
<td>2.07 ± 0.23</td>
<td>162 ± 13</td>
</tr>
<tr>
<td>M Con</td>
<td>79 ± 7*</td>
<td>2.14 ± 0.20</td>
<td>204 ± 29*</td>
</tr>
<tr>
<td>L</td>
<td>99 ± 12*</td>
<td>2.00 ± 0.29</td>
<td>274 ± 20*</td>
</tr>
<tr>
<td>M</td>
<td>92 ± 14*</td>
<td>2.13 ± 0.28</td>
<td>276 ± 25*</td>
</tr>
<tr>
<td>H</td>
<td>85 ± 9</td>
<td>2.14 ± 0.30</td>
<td>191 ± 21</td>
</tr>
</tbody>
</table>

*P < 0.05 vs the control group; #P < 0.05 vs the model control group; L: Low dose (20 mg·kg⁻¹·d⁻¹) of wheat peptides treated, M: moderate dose (100 mg·kg⁻¹·d⁻¹) of wheat peptides treated, H: High dose (500 mg·kg⁻¹·d⁻¹) of wheat peptides treated

Changes in NO level and iNOS activity of stomach

As shown in Fig. 3, there was a significant increase in the NO level in stomach obtained from the model control animals, compared with normal control animals (P < 0.05). The treatments with the low and moderate doses of wheat peptides significantly reduced the NO level, compared with the model controls (P < 0.05).

As shown in Fig. 4, there was a significant increase in the
activity of iNOS in the model control rats, compared with the normal control rats \( (P < 0.05) \). The low dose of wheat peptides significantly decreased the activities of iNOS in stomach, compared to the model control rats \( (P < 0.05) \).

**Changes in iNOS and µ-opioid receptor transcription of stomach**

As shown in Fig. 5, there was an increase in µ-opioid receptor transcription in stomach obtained from the model control animals, compared with normal control animals. The treatments with low and moderate doses of wheat peptides significantly reduced µ-opioid receptor transcription, compared with the model control animals \( (P < 0.05) \). As shown in Fig. 6, the low and moderate doses of wheat peptides significantly decreased the iNOS transcription, compared with the model control rats \( (P < 0.05) \).

**Discussion**

Proteases are often used to prepare peptides and characterize by a function of hydrolyzed protein, enzyme used, and conditions of hydrolysis \([20-22]\). The products are distinguished from the original protein mainly by their small molecular size \([23-24]\). Moreover, cleavage is usually accompanied by important structural rearrangements that tend to expose to the aqueous phase with some hydrophobic regions originally being buried within the protein molecule \([25-26]\). Peptides can therefore be very different from the parent proteins and may possess new nutritional, functional or biological properties. Enzymatic hydrolysates are thus used in food industry, parachemistry, and pharmacy \([27-28]\). Our data suggested that
wheat peptides obtained by the protease hydrolysis may be an effective tool for protecting stomach tissue against NSAID-induced damage.

A wide range of reactive oxygen species, including free radicals such as superoxide anion, hydroxyl radicals and non-free radical species such as hydrogen peroxide and singlet oxygen may form in the human body [98-30]. These radicals not only induce lipid peroxidation that causes cell and tissue damage, but also cause oxidative stress by oxidizing biomolecules, leading to cell death, tissue damage, and chronic diseases such as atherosclerosis, cancer, emphysema, cirrhosis, and arthritis [31-32]. More and more research results show that oxidative stress is a key reason for NSAIDs-induced gastric damage [33-35]. Antioxidants may reduce such oxidative damages and protect the normal structure and function of cells and mitochondria. Antioxidants can prevent some chronic diseases and neutralize the plethora of free radicals that are generated within the human body [36-37]. In recent years, bioactive antioxidative peptides (2 to 20 amino acid residues) have drawn the attention of researchers, due to their low molecular weight, easy absorption, high activity, and relatively high stability under different conditions [38]. It is reported that wheat peptides have potent antioxidant activity in vitro [9]. The present study found that the wheat peptides can play its antioxidant activity in vivo, even with the lower doses of wheat peptides, suggesting that wheat peptides are a biologically active substance besides nutrients. The mechanism is not very clear, and further mechanistic research is needed.

One of the major effects of oxidative stress is the production of superoxide ion radical O$_2^-$ and OH free radicals. These are powerful oxidizing agents produced during metabolism that can cause destruction of lipids, carbohydrates, proteins, and nucleic acids [29-30]. SOD, CAT, and GPx are among the key enzymes that defend the contents of cells against oxidative stress. SOD generates hydrogen peroxide from the O$_2^-$ (superoxide anion radical) [39-40], and CAT converts hydrogen peroxide to water and molecular oxygen, thus reducing their destructive effects [41-42]. In addition, GPx reduces hydrogen peroxide and lipid peroxides at the expense of glutathione to water and alcohols, respectively, to produce nonreactive molecules [43-44]. These enzymes may act in concert to reduce the damaging effects of ROS (Reactive Oxygen Species) and FR (Framework region) to levels that allow normal metabolic functions in cells and tissues. Many approaches have been investigated to increase SOD, CAT, and GPx to augment the demand levels required for defense against the ravages of oxidative stress. Our data suggested that wheat peptides could scavenge free radical by up-regulating the activity of SOD and GPx in vivo.

NF-κB has been identified as a transcription factor regulated by the intracellular redox status, which is activated by oxidative stress and induces the expression of a variety of proteins such as COX-2 and iNOS that function in the immunological and cellular detoxifying defense systems. It has also been shown to stimulate production of NO by activating of iNOS [45]. In fact, the oxidative stress induces NO in the tissue. Increased NO level leads to destruct the stomach tissue. When NO combines with superoxide radicals it becomes cytotoxic peroxynitrite anion. Raised levels of NO have been found in increased oxidative stress conditions, leading to cell degeneration and necrosis [46-48]. Our data showed significantly increased NO levels in the tissue of NSAID-induced stomach damage, which was consistent with the Bannwarth’s results [49]. The treatment with wheat peptides caused marked reduction in NO levels in stomach by down-regulating the activity and transcription of iNOS.

Opioid receptors can be subdivided into three major classes, µ-, δ- and κ-opioid receptors. Enkephalins and dynorphins are regarded as the endogenous ligands for the δ- and κ-opioid receptors, respectively. Huebner and Fukudome have described peptides isolated from the wheat protein, showing high opioid-like activity [50, 7]. The effects of µ-opioid receptors on stomach endocrine are well established [51]. Pharmacological studies suggest an effect of µ-opioid receptor agonists on endocrine in vivo and in vitro, on neurotransmitter release and on the peristaltic reflex [52]. Finally, the generation of µ-opioid receptor-knockout mice has established the influence of µ opioid receptors in delaying gastrointestinal transit [53]. However, little is known about the effects of the µ opioid receptor on the NSAID-induced stomach damage. From our results, intragastric administration of wheat peptides can decrease µ opioid receptor transcription in NSAID-induced stomach damage. Wheat peptides acted as a biological active substance, which played a role in the possible ways with the receptor was oversaturated, or inhibiting their receptor expression. Thus its mechanism is worth further study.

Based on our results, we conclude that the treatment with wheat peptides provides potential preventive effects on NSAID-induced stomach damage, by decreasing the oxidative stress and reducing in NO level.

References

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