The Protective Efficacy of Quercetin on Mefenamic Acid-Induced Gastric Mucosal Damage

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ABSTRACT
The objectives of this study were to determine the protective effect of quercetin on NSAIDs-induced gastric mucosal damage by measuring the level of superoxide radical, hydrogen peroxide (H2O2), protein carbonylation (PC), and advanced oxidation protein products (AOPPs). In this experiment, a gastric mucosa was taken from male rats (Rattus norvegicus). Samples then homogenized and divided into three groups with; T1 served as control which contains gastric mucosa homogenate only; T2 which contains gastric mucosa homogenate + 500 mg of mfenamic acid; and T3 which contains gastric mucosa homogenate + 500 mg of mfenamic acid + 250 mg/L of quercetin. After treatment, every 15-minute superoxide radical H2O2 PC, and AOPPs levels were estimated. The results revealed that mfenamic acid increased the rate of superoxide radical, H2O2 PC, and AOPPs formation, while quercetin decreases the rate of all parameters. These results indicated that mfenamic acid induce gastric mucosal damage through oxidative stress and inflammation mechanisms as can be seen from the increasing of superoxide radical, H2O2 PC, and AOPPs levels. Also, the results indicated that quercetin have a protective effect to gastric mucosa as can be seen from the decreasing of all parameters.

Keywords: Gastric Mucosal Damage, Inflammation, Mefenamic Acid, Oxidative Stress, Quercetin

INTRODUCTION
Gastric plays a pivotal role in the digestion of foods. Except for uncommon cases, this organ can expose to many noxious factors including hydrochloric acid, refluxed bile salts, and alcohol. This high resistance to injuries depends on a number of physiological responses elicited by the mucosal lining against potentially harmful luminal agents, as well as to the ability of rapidly repairing the mucosal damage when it does occur. Nevertheless, when these protective mechanisms are overwhelmed by injurious factors, a gastric mucosal lesion may develop. This lesion occurs when there is an imbalance between gastric offensive and protective factors. Several factors both exogenous and endogenous are responsible for a gastric mucosal lesion. These factors are Heliotobacter pylori infection, increase the production of gastric acids, pepsin, and stomach juices, tobacco, alcohol, caffeine, and certain types of medicine, especially the non-steroidal anti-inflammatory drugs (NSAIDs). The NSAIDs are widely used for the treatment of pain and inflammation in rheumatic disorders and osteoarthritis. These drugs are also used as antineoplastic agents and to prevent and treat ischemic heart disease. Among NSAIDs, mfenamic acid is one of the NSAIDs are widely used in the world. Like other NSAIDs, mfenamic acid inhibits prostaglandin biosynthesis. This is the basic common mechanism how the NSAIDs-induced gastric mucosal lesion. However, several mechanisms are proposed to the pathomechanism of NSAIDs-induced gastric mucosal lesion, including oxidative stress and inflammation. It was first report by Murata et al. They offer direct evidence that oxidative stress is involved in NSAIDs-induced gastric mucosal injury. Also, earlier findings in experimental animals show that lipid peroxidation was induced in rat stomach exposed to NSAIDs. Since oxidative stress is involved in NSAIDs mediated gastric mucosal lesion, the use of antioxidants might be useful to inhibit or reduced this effect. A group of antioxidants that is often suggested to be good candidates for antioxidant therapy due to their potential role in supporting health is the flavonoids. Generally, studies on flavonoids are inclined to focus on quercetin (QUR). QUR is widely available and easy to extract, isolate and detect. Commonly found in vegetables and fruits in the form of a glycoside. Also, QUR is accumulated in the GI tract, making this its main site of action. The result of the previous study shows that QUR reduced the oxidative stress, mitochondrial dysfunction...

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and cell death induced by indomethacin both in vitro and in vivo models of gastrointestinal damage. Also, QUR has been reported to prevent indomethacin-induced mitochondrial dysfunction through the antioxidant mechanism in Caco-2 cells. Based on the facts available in the literature, the present study was directed towards an assessment of the gastroprotective efficacy of quercetin, against mefenamic acid induced gastric mucosal lesion from the analysis of superoxide radical, hydrogen peroxide (H$_2$O$_2$), protein carbonylation (PC), and Advanced Oxidation Protein Products (AOPPs) levels.

MATERIAL AND METHODS

Animals and Homogenate Preparation
Male rats (Rattus novergicus) weighing 200–250 gram with 2-3 months old were obtained from the Abadi Jaya farm at Yogyakarta, Indonesia, in healthy condition. The experiment was approved by the Ethical Committee of the Lambung Mangkurat University, South Kalimantan, Indonesia. Animals were fed under standard conditions and acclimatized with a 12 hours light/dark cycle. The animals were sacrificed by surgical procedure and the gastric was opened. The gastric along the greater curvature, and rinsed with PBS. Each homogenate was collected and stored at–80 °C until determination of SOD activity, H$_2$O$_2$, MDA, and PC levels.

Experimental Models

Homogenate samples were divided into 3 groups (1 control group and 2 treatment groups). Control (T1) group: homogenate only; Treatment 1 (T2) group: homogenate + 500 mg of mefenamic acid; and Treatment 2 (T3) group: homogenate + 500 mg of mefenamic acid + 250 mg/L QUR. Each solution then incubated at 37°C for 60 minutes, and every 15 minutes the SOD activity, H$_2$O$_2$, PC, and AOPPs levels were estimated.

Superoxide radical level analysis

The superoxide radical level was measured by the method of Misra and Fridovich with slight modifications. Samples were added to 0.800 ml of carbonate buffer (100 mM, pH 10.2) and 100 µl of adrenaline 3 mM resulted in adenochrome. Superoxide radical levels are calculated based on adenochrome which formed in solution by measuring the absorbance at 480 nm.

H$_2$O$_2$ level analysis

The H$_2$O$_2$ level was calculated by the FOX2 method with slight modification. Solutions measured spectrophotometrically at $\lambda = 505$ nm. Standard and test solutions consisted of 1 M H$_2$O$_2$ 200 µL and 200 µL serum, respectively, with the addition of 160 µL PBS pH 7.4, 160 µL FeCl$_3$ (251.5 mg FeCl$_3$ dissolved in 250 ml distilled water) and 160 µL o-phenanthroline (120 mg o-phenanthroline dissolved in 100 ml distilled water) for both solutions. The composition of the blank solution was identical to that of the test solution, except for the absence of FeCl$_3$ in the blank. Subsequent to preparation, all solutions were incubated for 30 minutes at room temperature, then centrifuged at 12,000 rpm for 10 minutes, and the absorbance of the standard (As), test (Au) and blank (Ab) solutions measured at $\lambda = 505$ nm, using the supernatant of each solution.

PC level analysis

PC was calculated by measuring the total protein carbonyl content. The total protein carbonyl content was determined by colorimetric method. The liver homogenate (0.5ml) was pipetted into 1.5 ml centrifuge tube and 0.5 ml of 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl was added and allowed to stand at room temperature for 1 hour, with vortexing every 10-15 minutes. Then, 0.5ml of 20% Trichloroacetic acid was added followed by centrifugation. The supernatant was discarded and the pellets were washed 3 times with 1 ml ethanol-ethyl acetate (1:1) to remove free reagent. The obtained precipitated protein was redissolved in 0.6 ml guanidine solution. Carboxyl content was calculated from maximum absorbance (390nm).

AOPPs level analysis

AOPPs measurement was made by spectrophotometric methods as describe by Witko-Sarsat et al., with slight modification. Briefly, AOPPs were measured by spectrophotometry on a microplate reader and were calibrated with chloramine-T solutions that in the presence of potassium iodide at 340 nm. In test wells, 200 ml of plasma diluted 1/5 in phosphate buffer solution were placed on a 96-well microtiter plate and 20 ml of acetic acid was added. In standard wells, 10 ml of 1.16 mol potassium iodide was added to 200 ml of chloramine-T solution (0–100 mmol/l) followed by 20 ml of acetic acid. The absorbance of the reaction mixture is immediately read at 340 nm on the microplate reader against a blank containing 200 ml of phosphate buffer solution, 10 ml of potassium iodide, and 20 ml of acetic acid. The chloramine-T absorbance at 340 nm being linear within the range of 0 to 100 mmol/l. AOPP concentrations were expressed as µmol/l of chloramine-T equivalents.

Data analysis

The results were expressed as mean±SE for two replicates. The data was analysed between each parameter level and incubation time. For analyzing the data, Microsoft Excel 2010 was used and was examined by simple linear correlation. Furthermore, correlation chart that was generated for each treatment compared to one another.

RESULTS

We first measured H$_2$O$_2$ formation in each treatment. For T1 group, H$_2$O$_2$ generation is lower than the T2 group in all time of incubation. The T2 group has the highest levels compared to another group of treatments in all time of incubation. The second parameter that we analysed in this present study is superoxide radical level. The result shows in figure 2. From figure 2, it can be seen that the level of superoxide radical has positive correlation in all group of treatments. T1 seems to have a lower superoxide radical
level than T2 group in all time of incubation except at minute 60. T3 seems can decrease the level of superoxide radical till below the T1 group. The third parameter that we analysed in this present study is PC level. The result shows in figure 3. From figure 3, it can be seen that the lowest rate of PC level is in the T1 group and the highest
Figure 5: ROS, PC, and AOPPs formation by mefenamic acid. Mefenamic acid causes the formation of •O₂ in gastric mucosa. •O₂ dismutated to form hydrogen peroxide (H₂O₂). H₂O₂ is converted to water (H₂O) and oxygen (O₂) by catalase (CAT), and peroxidase (Px). In the presence of transition metals such as copper and iron, further reactions take place, e.g., through the Haber–Weiss mechanism or the Fenton reaction to give up radical hydroxyl (•OH). All reactive oxygen species (ROS) that produced by mefenamic acid promote a further reaction with gastric mucosa protein resulted in protein carbonylation (PC). Also, all these ROS-induced the formation of advanced oxidation protein products (AOPPs) which is a marker of inflammation in gastric mucosa.

is in T2 group. T2 can increase PC level in all time of incubation period compared to the T1 group, while T3 also increase the PC level compared to T1 group. However, if we compared the rate of PC level between T2 and T3 group, is seems T3 can decrease the rate of PC level in all time of incubation period. The last parameter that we investigated in this present study is AOPPs. The result is shown in figure 4. AOPPs level shows a positive correlation with incubation time in all group of treatments. The AOPPs level in T2 group is higher than T1 and T3 group in all time of incubation, while in the T3 group is lower than T1 and T2 group.

DISCUSSION
Recently we reported that mefenamic acid could increase the generation of superoxide radical and H₂O₂ in gastric mucosal homogenate (figure 5). As far as we know there have been no investigations of the association between mefenamic acid treatment and superoxide radical or H₂O₂ levels. However, Chattopadhyay et al. showed that indomethacin treatment induced oxidative stress in rat gastric mucosa by the irreversible inactivation of gastric peroxidase. Also, another evidence indicating that NSAIDs could induce gastric mucosal damage through the releasing of oxygen metabolites17. It’s been widely accepted that NSAIDs could induce the gastric mucosal damage through the formation of reactive oxygen species (ROS). There are several mechanisms of how NSAIDs can trigger the formation of ROS5.

These mechanisms are
NSAIDs accumulate in gastric epithelial cells through ‘ion trapping’, with subsequent uncoupling of mitochondrial oxidative phosphorylation and inhibition of the electron transport chain. This results in depletion of intracellular ATP, cellular Ca²⁺ toxicity, and generation of reactive oxygen species (ROS) like superoxide and hydroxyl radicals5.

NSAIDs, indomethacin (IND) was found to bind to a site adjacent to the complex I and ubiquinone of mitochondrial electron transport chain. This binding generates ROS and ROS, in turn, inactivate mitochondrial aconitase. Inactivation of mitochondrial aconitase results in the production of free iron, which in turn generates more mitochondrial •OH5. NSAIDs could induce the generation of proinflammatory cytokines which activate NADPH oxidase resulted in ROS formation18. NSAIDs induces ischemia in the mucosal region through suppression of prostaglandin synthesis and decreased blood flow. Under ischemic conditions the mitochondrial electron transport chain remains in a relatively reduced state, favoring leakage of electrons from some specific sites of the electron transport chain, mainly from complex I and complex III, leading to partial reduction of O₂ to generate •O₂, which is dismutated to form H₂O₂ by superoxide dismutase19. As we know, in general, NSAIDs may induce the formation of ROS in gastric mucosa through several mechanisms, as mentioned in the previous paragraph. However, the mechanism only applies in general to NSAIDs, and did not explain specifically about mefenamic acid. This study has shown that mefenamic acid may also increase the formation of ROS in gastric mucosa, and we propose a new mechanism of how mefenamic acid can increase it. The mechanism is presented in Figure 5. It is well known that oxidative stress could trigger the PC (figure 5). PC is the most frequent type of protein modification in response to oxidative stress, is thought to be irreversible and destined only to induce protein degradation in a nonspecific manner20. It is in line with the result of this present study. The result indicated that NSAIDs increase PC. This may
be due to the increased formation of ROS, as mentioned earlier. ROS can react directly with the protein or they can react with molecules such as sugars and lipids, generating products (reactive carbonyl species, RCS) that then react with the protein. Direct oxidation of proteins by ROS yields highly reactive carbonyl derivatives resulting either from the oxidation of the side chains of lysine, arginine, proline, and threonine residues—particularly via metal-catalysed oxidation—or from the cleavage of peptide bonds by the α-amidation pathway or by oxidation of glutamyl residues. ROS-mediated protein damage also can be seen from the formation of AOPPs. AOPPs are predominantly albumin and its aggregates, which are generated the following exposure to ROS from activated phagocytes. Result of this present study revealed that NSAIDs increase the formation of AOPPs (figure 5). Also, this may be due to the increased formation of ROS. AOPPs are also believed to be more closely related to inflammation. AOPPs could bind to the receptor for advanced glycation end products (RAGE). These interactions trigger the ROS formation, with activation of nuclear factor NF-kB and release of pro-inflammatory cytokines. Since AOPPs closely related to inflammation, the increasing of AOPPs level in gastric mucosa may be indicated the inflammation in gastric mucosa by menameic acid (figure 5). The results also suggested that QUR has a protective effect on NSAID-induced gastric mucosal damage. QUR can decrease the level of all measured parameters at all time of incubation. The protective effect of quercetin may be due to the antioxidant activity of QUR. QUR ability to scavenge free radicals and bind to transition metal ions makes it considered a strong antioxidant. This is due largely to the pharmacophores found within QUR including a catechol group found in ring B. QUR is the most potent scavenger of radical superoxide, and ONOO− within the flavonoid family and its qualities as an antioxidant make QUR a strong lipid peroxidation inhibitor. In addition to its antioxidant properties, QUR also increases glutathione concentrations of which can inhibit free radical formation.

In conclusion, the present study demonstrated that menameic acid induced gastric mucosal damage through oxidative stress and inflammation mechanisms as can be seen from the level of superoxide radical, H₂O₂, PC, and AOPPs. Also, the present study demonstrated that QUR has a protective effect to gastric mucosa which can be seen from the decreasing of superoxide radical, H₂O₂, PC, and AOPPs levels in gastric mucosa.

CONFLICT OF INTEREST
We declare that we have no conflict of interest.

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