Research into the effect of proton pump inhibitors on lungs and leukocytes.

ORHAN OZATİK[1], Fikriye Ozatik[1], Yasemin Teksen[1], İlknur Dağ[2], Suna Saygili[1], and Ahmet Kocak[1]

[1]Kütahya Health Sciences University - Evliya Çelebi Campus
[2]Eskisehir Osmangazi University

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Abstract

Backround and Purpose. Proton pump inhibitors (PPI) are the most commonly used medication in the world. They are prescribed as an effective treatment choice for gastrointestinal system diseases linked to hyperacidity, especially. Many publications in recent times have reported significant side effects. However, there are insufficient studies about the topic of the mechanism for these side effects. Experimental Approach. Rats were divided into 3 groups of control, a group administered H2 receptor blockers and a group administered PPI (n: 8). Medications were administered for 30 days intraperitoneal. After 30 days, rats were euthanized and lung tissue was obtained. Lung were stained for immunohistochemical Catalase, Superoxide Dismutase, Glutation Peroxidase, Myeloperoxidase and toluidine blue and investigated with a light microscope. Transmission Electron Microscopy (TEM) was used to investigate lung tissues and neutrophil leukocytes. Additionally, lung tissue had biochemical H2O2 levels researched. Key Results. H2O2 amounts, produced by lysosomes with important duties for neutrophil functions in lung tissues, were found to be statistically significantly reduced in the group administered PPI. Results of investigations of preparates obtained with immunohistochemical staining observed increases in antioxidant amounts in the PPI group. Investigation with TEM identified more inflammation findings in the lung tissue from the group administered PPI compared to the control group and the group administered H2 receptors. Conclusion and Implications. In conclusion, we identified long-term PPI use disrupts neutrophil leukocyte functions in lung. All clinicians should be much more careful about PPI use.

1-INTRODUCTION

Proton pump inhibitors (PPI) are among the most commonly used medications in the world. With the initiation of use of the first PPI, omeprazole, at the end of 1980, PPIs were proven to be an effective treatment choice for a variety of diseases linked to acid including gastroesophageal reflux disease, peptic ulcer disease, Helicobacter pylori eradication treatment, dyspepsia and stress (Luo., Fan et al., 2018). The use of PPIs rose by 450% toward the end of the 1990s (Guda., Noonan et al., 2004). Currently we see many publications related to unnecessary and increasing rates of PPI use. A study in Spain observed high frequency of PPI use during hospital admission of patients and in the period after discharge (R). Additionally, in the period after discharge, PPI use continued with no indications for nearly 3-6 months. Use with the aim of preventing possible harmful effects of a variety of medications on the stomach is excessive. PPI group medications are known as stomach protectors among the public. People participating in a survey in research encompassing many countries around the world identified nearly 30% had acid indigestion, heartburn and reflux complaints (Luo., Fan et al., 2018). According to the results of the same study, the medications used to treat these people were mainly proton pump inhibitors. People using PPIs for many years are told that these medications only affect the stomach and have no effect on other tissues or organs and that they can use them comfortably. As a result, excessive amounts of use are present.

In the past PPIs were frequently used for patients being treated in intensive care units with frequent multiple drug use. During use by these types of patients, it was predicted to affect patient mortality, so the use of H2 receptor blockers was recommended in these cases (Hamai., Iwamoto et al., 2018). Deaths occurring due to the use of PPIs in patients treated in intensive care occurred due to bacterial proliferation linked to the stomach pH becoming alkali and bacteria reaching the lungs causing infections, with patients proposed to have died due to lung infections (Hamai., Iwamoto et al., 2018; ; Garvey., McCambley et al.,1989). However, the H2 receptor blockers used as alternative treatments also make the stomach pH alkali. Though both medications increase stomach pH, the effect mechanisms are different. The effects of PPIs are shown through acid pH. If the pH of body cells and tissues do not remain within physiologic limits, it is not compatible with life; as a result, if pH doesn't fall PPIs do not become active and it is proposed they have no effects outside the stomach. It is not fully clear which mechanism forms a variety of side effects of PPIs. In vitro studies have revealed reduced neutrophil bactericidal activity caused by omeprazole (Zedtwitz-Liebenstein., Wenisch et al., 2002). Different to previous explanations related to pneumonia occurring as a result of PPI use and resulting deaths, our thoughts are as follows; the most important effect mechanism in the struggle between leukocytes, especially neutrophil leukocytes, with bacteria are degradation and digestion of phagocytic bacteria by lysosomes. The pH within lysosomes is very acidic. For degradation and digestion of bacteria within lysosomes, hydrogen peroxide (H_2O_2) should form within lysosomes and the H_2O_2 should later be transformed into hypochlorous acid (HOCl). For creation of H_2O_2 in lysosomes, H must be taken inside the lysosomes. In order for H to be taken into lysosomes, a proton pump must be used. After long-term PPI use, the proton pumps in lysosomes are inhibited which means the struggle between neutrophil leukocytes with bacteria will not provide results.

In light of these findings, in our study we attempted to research the possible effect mechanisms of PPIs on some tissues and cells outside the stomach and to reveal the cause of side effects proposed to be caused by proton pump inhibitors and our hypothesis. With this aim, lung tissues from experimental animals were stained with catalase (CAT), myeloperoxidase (MYP), superoxide dismutase (SOD) and glutathione peroxidase (Gpx) stains in an attempt to observe the activities of these enzymes. Additionally, lung tissues were investigated with an electron microscope to assess morphologic changes. These enzymes effective on oxidative stress and antioxidant mechanisms are some of the parameters required to prove our thesis. SOD is a 32 KDa hemodimeric metalloenzyme basically found in plasma, nucleus and cytosol (Chen., Watson et al., 2017). It catalyzes the differentiation of a catalytic copper ion and superoxide radical into dioxygen and H_2O_2. Extracellular SOD is released and synthesized by fibroblast cells, glial cells, and endothelial cells. Lung tissue has high extracellular SOD levels. SOD is the only antioxidant which can inactivate enzymatic free oxygen radicals at extracellular level. As a result, extracellular SOD undertakes important duties in terms of protecting against diseases like oxidant injury, inflammation and fibrosis (Gao., Kinnula et al., 2008). SOD increases efficacy when the organism experiences increased oxidant stress. Especially in clinical situations with reduced effect of antioxidant systems, SOD activity increases (Rahimi., Rakhshandeh et al.,2019; Limon Pcheco and Gonsebatt., 2009). In our study, in parallel with this knowledge, SOD activity was observed to increase in lungs.

Gpx is the most effective enzyme in endothelial cells, especially in lung (Cheeseman and Slater.,1993). Free radicals are highly reactive species damaging DNA in proteins, lipids and carbohydrates causing structural cell injury and apoptosis. Exogenous antioxidant enzymes like CAT, SOD, Gpx, MYP and thiol groups protect against oxidative stress injury caused by free radicals (Sheng., Abreu et al., 2014).

Additionally, we researched whether the H_2O_2 levels found in lung tissues were different between the groups. Clinical research in the recent period has shown significant side effects of this medication group; however, it has still not been fully revealed which effect mechanism causes the occurrence of these side effects.

2-MATERIAL AND METHOD

2.1-Experimental animals and groups:

This study used 24 Wistar Albino male rats weighing 240-280 g. Animals were housed in fixed limits at

room temperature (24±2°C) with 55%±15 relative humidity and 12 hour/12 hour light-dark cycles. Water and standard rat feed were given ad libitum. Care was taken that all procedures related to animals were completed in accordance with national and international regulations related to animal experiments. The study was completed in Kutahya Health Sciences University Experimental Animal Breeding Research and Application Center, Dumlupinar University Advanced Technology Design, Research and Development Center and Eskisehir Osmangazi University, Central Research Laboratory, Application and Research Center. The study was completed in two stages. The first study received ethics committee permission from Dumlupinar University Animal Experiments Local Ethics Committee. Later, in order to more clearly prove the hypothesis, tissue obtained from the previous study were studied with an electron microscope and for this second ethics committee permission was obtained from Kutahya Health Sciences University Animal Experiments Local Ethics Committee.

The first stage of the study was supported by Dumlupinar University -SRP and the second stage was supported by Kutahya Health Sciences University -SRP.

Experimental animals were divided into 3 groups;

Animals in the first group (Control group) were used as controls, were administered a single dose of ip physiological saline (PS) every day for thirty days and were euthanized at the end of the month with lung tissue removed.

Animals in the second group (PPI group) had a single dose of ip PPI (pantoprazole 40 mg, Sandoz/Turkey) administered every day for thirty days and were euthanized at the end of the month with lung tissue removed.

Animals in the third group (Ra Group) had a single dose of ip H2 receptor blocker (Ranitidin 50 mg/2 ml, Deva/Turkey) administered every day for thirty days and were euthanized at the end of the month lung tissue removed.

Lung samples obtained in the study were assessed for histopathologic and biochemical changes.

2.2-Histologic Investigation

The removed lung tissues were divided in 2 for histopathologic investigation. Some was fixed in 10% neutral formalin solution. Standard histologic techniques were applied. Later they were submerged in paraffin and sectioned to 5 µm thickness. Lung was stained with CAT, SOD, Gpx and MYP staining and investigated with a light microscope. The section allocated for electron microscope investigation was fixated in 2.5% glutaraldehyde for 24 hours at 4 . Later, secondary fixation was performed in 1% osmium tetroxide at room temperature in a rotator. Tissues were washed 3 times in buffer solution, then passed through an ethyl alcohol series at 4 twice. Propylene oxide was used for transparency. Tissues were submerged in araldite the next day. The obtained blocks were sliced with an ultramicrotome and prepared for investigation. Investigation with transmission electron microscopy (TEM) assessed histopathologic changes occurring in lung tissue and especially, changes in neutrophil leukocytes.

All histologic assessments were performed by two histologists blinded to the groups.

Immunohistochemical staining in the groups was determined according to intensity (i) as (0): no staining, (1): weak, (2): moderate, (3): strong, and (4) severe staining.

Immune staining histologic scoring system (histologic score: H-score) was calculated with the following equation: H-SCORE: Pi (i+1). 1. The H-SCOR was separately obtained for lung tissue from the multiplication of staining intensity of stained cells with percentages (Mc Carty., Miller et al., 1985).

2.3-Biochemical Investigation

After lung were removed from animals, they were homogenized in a mechanical homogenizer in 50 mmol/L phosphate buffer (pH 7.40) for biochemical investigation. Homogenates were centrifuged at 10,000 g for 15 minutes at 4 , with supernatants stored at -80 .

2.3.1-Hydrogen Peroxide Analysis

A hydrogen peroxide kit (ab102500, abcam) was used according to the manufacturer's instructions and spectrophotometric measurements were performed at 570 nm. H_2O_2 analysis forms one of the most important parameters in the study. This method will be assessed to reveal the effect mechanism of the drugs and to prove our hypothesis.

2.4-Statistical analysis

Results are given as mean ± SEM. Data were assessed with the one-way analysis of variance (ANOVA) test (post hoc Dunnett test) with the SPSS program. The Kruskal-Wallis test (post hoc Dunn's method) was used to compare histopathologic results. $P<0.05$ was accepted as significant.

3-RESULTS

3.1-Histologic Investigation Results

3.1.1-SOD

According to results obtained for H-scores for immunohistochemical staining, lungs were observed to have SOD activity increased by a significant degree in the PPI group compared to the control group ($p<0.002$). Though there was a significant increase in the Ra group compared to controls, it was not as much as for PPI ($p<0.002$) (Figure 1). When this result is compared with immunohistochemical pictures, strong staining was identified in the PPI group, with less staining observed in the Ra and control groups (Figure 2-A). According to this result, PPIs can be said to increase SOD activity more compared to H2 receptor blockers.

3.1.2-Gpx

Similar results are encountered for H-score as seen in the SOD group. Gpx activity increased in the PPI group ($p<0.002$), with less activity observed in the Ra group ($p<0.002$) (Figure 1). When glutathione peroxidase staining is investigated on immunohistochemical pictures, strong immunoreactivity was observed in the PPI and Ra groups, with moderate intensity staining observed in the control group (Figure 2-B). PPIs were concluded to be more effective on glutathione peroxidase activity in lung compared to H2 receptor blockers.

3.1.3-MYP

H-scores showed that MYP activity was significantly elevated in the PPI group compared to the control group ($p<0.005$). However, the same effect was not observed in the Ra group (Figure 1). When the PPI and Ra groups are compared, MYP values were lower in the Ra group compared to the PPI group ($p<0.005$) (Figure 1). When assessed in terms of myeloperoxidase staining, the PPI group was observed to have moderate severity immunoreactivity, while the Ra and control groups were identified to have less intense staining (Figure 2-C).

3.1.4-CAT

When the H-scores for catalase activity are examined, the means for the groups were observed to be very high. While significant catalase activity was observed in comparing the PPI group with the control group ($p<0.002$), the mean in the Ra group was significantly low compared to the PPI group ($p<0.002$) but was significantly high when compared to the control group ($p<0.002$) (Figure 1). When the experimental groups are compared in terms of catalase staining, the PPI group had severe immunoreactivity observed, while the Ra group had strong immunoreactivity. The control group was observed to have low-moderate intensity immunoreactivity compared to these two groups (Figure 2-D).

3.1.5-Lung Toluidin Blue Staining

According to light microscope data obtained from control rat lung samples on half thin sections with toluidine blue staining, pulmonary alveoli with normal spread were observed. Alveolar epithelium had normal appearance and thin edges. These structures were divided by fine alveolar septa.

According to light microscope data from the PPI group, increased and abnormal distribution of collagen fibers were observed around blood capillaries. The increase in erythrocyte amounts was notable. Occasionally closed and narrowed alveoli were observed. Alveolar macrophage increase was observed in the alveolar lumen.

According to light microscope data obtained from the group administered H2 receptor blockers, alveoli and alveoli walls had abnormal and irregular structures. There was very excessive alveolar narrowing. Thickening of alveolar walls was observed (Figure 3).

3.2-TEM Investigation Results

In our study, TEM images obtained from the control rat lung samples are presented in Figure 4 (A-D). According to these data, Type 1 and Type 2 alveolar epithelial cells and basal lamina in the alveolar wall have a healthy morphology. In general, the alveolar epithelial wall structure has shown regularity. Type 1 pneumocytes with regular oval nucleus in normal sizes and shapes were detected. Intense peripheral chromatin was seen in the nucleus of these pneumocytes. Type 2 pneumocytes with normal peripheral chromatin and regular nucleus structure were also observed. Vacuols were also found in some cells.

In TEM data obtained from PPI treated groups (Figure 5 A-D), the most prominent finding was detected as alveolar epithelial thickening and irregularity. The increase in the amount of erythrocytes is remarkable. The peripheral chromatin density in cell nucleus has increased very much and a few erythrocyte debris has been observed in the alveolar lumen. In addition, lamellar body increase was observed in Type 2 pneumocytes. Nucleus and lamellar body damage and fusion were detected in some type 2 cells. Mitochondrial swelling and cristae damage were also observed in some cell cytoplasm. Occasional vacuole formations and electron dense inclusions were observed. In some nucleus structures, advanced membrane ondulations and pyknotic appearance were observed. Cellular debris has been observed in some areas of the alveolar lümen.

In TEM micrographs obtained from H2 receptor blocker treated groups (Figure 6 A-D), alveolar epithelial thickess and integrity loss were observed. In general, the size of the type 2 pneumocytes is larger than the control group, and lamellar bodies was found to be irregular and damaged rather than regular oval appearance. Apical microvillus structures are relatively well preserved in some cells. However it has shown irregularity, rupture and shortening in some areas. In some cells, dense peripheral chromatin and nucleus ondulations were determined. Collagen fibers are evident, mitochondria are relatively healthy, and vacuol formations have been observed.

4-Biochemical Investigation Results

4.1- H_2O_2 Analysis

Fluorometric measurements identified a significant fall in the PPI group. This result, though not definite, shows very low levels in the lung tissue of the group administered PPI when mean H_2O_2 values are taken as the basis. While the mean in the control group was 79.33, the mean in the PPI group was identified as 35.67 (Figure 7).

5-DISCUSSION

Lysosomal acid hydrolases assisting lysosomes found in leukocytes in lungs in performing their duties are activated in acidic pH. The necessity of acidic pH for lysosomal hydrolases provides double protection against uncontrolled digestion of cytosol content. Even if the lysosomal membrane degrades, the released acid hydrolases will be inactive in the neutral pH of the cytosol. To preserve internal acidic pH, it is necessary for lysosomes to concentrate active H+ ions. The active transport of these ions into the cell is assisted by proton pumps in the lysosomal membrane. The most important effect mechanism in the struggle of leukocytes, especially neutrophil leukocytes, with bacteria is degradation and digestion of phagocytized bacteria with lysosome (Cooper., 2000). For degradation and digestion of bacteria within lysosomes, H_2O_2 must be formed within the lysosome and the H_2O_2 must later transform into HOCl acid.

The results of our study investigated preparations obtained with SOD, Gpx, MYP and catalase immunohistochemical staining of lung tissue and observed a significant degree of elevation in the PPI group compared

to the control group in H-scores. The H_2 receptor blocker of ranitidine (Ra) was not as effective as PPI; however, a significant increase was observed in the Ra group compared to the control group. Pictures obtained from groups with histochemical staining and toluidine blue staining showed increased infiltration in lung and histopathologic changes.

SOD is a 32 KDa hemodimeric metalloenzyme basically found in plasma, nuclei and cytosol (Chen., Watson et al., 2017). It catalyzes the separation of a catalytic copper ion and superoxide radical to dioxygen and hydrogen peroxide (H_2O_2). SOD also increases activity when the organism experiences increased oxidant stress. Especially in clinical situations with reduced effect of the antioxidant systems, SOD activity increases. In our study, in parallel with this information, SOD activity appeared to be increased in lung. The reason for the increase in SOD activity is the reduction occurring in oxidative stress, while we think another reason is catalysis of H_2O_2 formation expected to form in lysosomes. The changes in H_2O_2 amounts support this. The H_2O_2 amount obtained in the PPI group (35.67) was very low compared to the H_2O_2 amount obtained in the Ra group (88.67) and this was statistically significant. In both groups, in spite of histopathologic injury to the lungs, the statistically significant difference in H_2O_2 amounts found in lung tissue show that PPIs prevent sufficient formation of H_2O_2 in lysosomes found in neutrophils.

In our study, we saw significant degrees of elevation in SOD, CAT, MYP and Gpx activity in addition to antioxidant enzymes in the control group. A study by Rahimi et al. (Rahimi., Rakhshandeh et al., 2019) induced acute respiratory distress syndrome (ARDS) in rats and examined the anti-inflammatory and antioxidant activity of 'Portulaca Olerecea' extract and here they identified SOD, CAT and MYP activity increased after addition of Portulaca Olerecea extract. The researchers attempted to determine antioxidant enzyme levels with the aid of commercial kits using serum samples. A study by Hackert et al. examined the effects of pantoprazole on acute pancreatitis and in conclusion they saw inflammation was suppressed. The researchers concluded that pantoprazole had anti-inflammatory effects and reduced the progress of acute pancreatitis. The researchers examined MPO activity in studies and observed MPO activity increased with pantoprazole (Hackert., Tudor et al., 2010). However, a study from 6 years before in 2004 examined the in vivo anti-inflammatory properties of pantoprazole and concluded that it did not have anti-inflammatory features (Becker., Maróstica et al., 2004). The antioxidant enzyme activity was increased in the groups given PPI and Ra. The reason for this was not the increase in anti-inflammatory and antioxidant levels due to PPI and ranitidine, it is activation of the defense mechanism of the body against inflammation and oxidative stress induced by them. The result is an increase in the antioxidant levels in the body.

PPIs are the first medications chosen for treatment of diseases related to acid secretion. Long-term use is observed especially in situations like gastroesophageal reflux. Apart from this, we are against excessive prescriptions in unnecessary situations. In these situations, PPIs affect the lysosome and lysosomes will not be able to fulfil their duty to destroy bacteria and will cause increased lung infection.

In our study results, we saw the thesis related to the proliferation of bacteria linked to increased stomach pH and later pneumonia causing death, proposed as the reason for deaths occurring as a result of PPI use among patients in intensive care especially, was deficient. Our H_2O_2 results in lung tissue showed that lysosomal function disorder induced in neutrophil leukocytes in lung tissue worsened progression of disease.

In conclusion, PPIs do not just affect parietal cells found in the stomach. They may affect proton pumps in other tissues and cells in the organism. As a result, the use and indications for PPI should be reassessed considering more possible side effects than previously determined.

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FIGURE CAPTION

Figure 1: Effects of Proton Pump Inhıbıtor tretament on histological parameters in rats. Date are as given ±SEM. *p< 0.05vs cotrol group, +p<0,05 vs PPI group ANOVA (n: 8).

SOD: Superoxide Distutase; **Gpx:** Glutatione Peroxidase;**MYP:** Myeloperoxidase; **CAT:** Catalase **PPI:**Proton Pump Inhibitors; **Ra:** Ranitidin

Figure 2: Reprasantive images of immunohistochemical staining.**A:** Superoxide Dismutase; **B:** Glutathione Peroxidase;**C:** Myeloperoxidase; **D:** Catalase.

Figure 3: Reprasantive images of lung Toluidin Blue Staining.**PPI:** Proton Pump Inhibitors; **Ra:** Ranitidin.

Figure 4: Reprasantive images of lung TEM Control Staining.

Figure 5: Reprasantive images of lung TEM PPI Staining.

Figure 6: Reprasantive images of lung TEM Ra Staining

Figure 7: Effects of Proton Pump Inhibitor and H_2 receptor blocker tretament on histological parameters in rats. Date are as given ±SEM. *p< 0.05 vs cotrol group, +p<0,05 vs PPI group ANOVA (n: 8).

PPI: Proton Pump Inhibitors; **Ra:** Ranitidin.