Effect of the chronic treatment with a proton pump inhibitor, rabeprazole, on the complete blood count in mice

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Abstract: Proton pump inhibitors are now one of the most widely used classes of drugs. This class of medications has gained popularity for acid suppression because they inhibit the last step in gastric acid secretion regardless of the stimulus for acid secretion and can be dosed once a day in most patients. Proton pump inhibitors have proven to have a very favorable safety profile and it is unusual for a patient to stop these drugs because of side effects. However, because of the increasing numbers of patients who are chronically taking proton pump inhibitors for gastroesophageal reflux disease and a number of other common persistent conditions, the long-term potential adverse effects are receiving increasing attention. One area that is receiving much attention and generally has been poorly studied is the long-term effects of proton pump inhibitors on the platelet count. In the current study, we investigated the effect of chronic administration of one of the PPIs, rabeprazole, on the complete blood count focusing on the platelet count in mice. Our results showed a significant drop in platelet count from 1783 ± 65 in the normal mice to 1345.6 ± 103.75 in rabeprazole-treated mice. A significant leucopenia was also observed in the treated group. However, there was no significant difference observed regarding other measured blood parameters among the two groups.

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1. Introduction:

The two major types of acid-related disorders are peptic ulcer disease and gastroesophageal reflux disease (GERD). The target for treatment was and still is reduction of gastric acidity (Hartmann *et al.*, 1996). However, despite clinical and commercial success, histamine-2 receptor antagonists (H_2RA) have several pharmacologic limitations that are increasingly apparent in the clinical setting. The H_2RA are less effective for the management of GERD and gastrointestinal bleeding than for healing of peptic ulcer disease, and the rapid development of tachyphylaxis limits their usefulness for long-term maintenance treatment or high-dose intravenous use (Teyssen *et al.*, 1995). For these reasons, the development and introduction of the first proton pump inhibitor (PPI), omeprazole, for the management of acid-peptic disorders marks one of the great success stories in gastroenterology. PPIs are substituted benzimidazole derivatives that have potent antisecretory effects on gastric acid (Eslami and Nasseri-Moghaddam, 2013). They block the terminal step in acid production by irreversibly inhibiting the function of the hydrogen-potassium adenosine triphosphatase present on the luminal aspect of parietal cell membranes in the stomach (Dajani., 2000). Their mechanism of action is unique as they accumulate in the highly acidic canalicular space of the active parietal cell, where the pH is less than 2.0. At this pH, they are converted to the active form of the drug, which then covalently binds to one or more cysteines that are accessed from the luminal surface of the pump (Shin and Sachs, 2002). Because of covalent binding, and inhibitory effects lasting much longer than their plasma half-life, no tolerance has been observed with this class of medications (Sachs *et al.*, 2010). However, they require the presence of acid secretion for accumulation and activation; hence their action is meal-dependent. Moreover, they have a relatively short plasma half-life of about two hours. Given this mechanism of action, the effect on acid secretion is cumulative, increasing to steady state after three to five days of administration, because pumps that are non secreting will not be inhibited whereas inhibited pumps will stay inhibited (Sachs and Shin, 2008).

PPIs are now indicated for the treatment of gastric or duodenal ulcer, dyspepsia, non steroidal anti-inflammatory drugs induced ulcer, for the treatment and maintenance of GERD, and for the eradication of helicobacter pylori and hypersecretory disorders, such as Zollinger Ellison syndrome (Dekkers *et al.*, 1999; Norton *et al.*, 1999; Agrawal *et al.*, 2000; Wolfe and Sachs, 2000). The success of these drugs, with sales totaling $13.6 billion worldwide in 2009, (Gatyas., 2010) is not just a result of their potency and effectiveness in improving symptoms and complications of acid-peptic disease.

Their safety among pharmacologic agents has been unparalleled (Madanick., 2011). However, recent reports have questioned the long-term safety of PPIs and evidence is mounting that these medications can lead to some troublesome and even serious side-effects. Furthermore, these drugs are too often used in patients who have no valid indication for them, exposing these patients to unnecessary risks. (Zink *et al.,* 2005; Heidelbaugh and Inadomi, 2006).

The collective body of information overwhelmingly suggests an increased risk of infectious complications. Laheij *et al.* (2004) showed that the adjusted relative risk for community-acquired pneumonia among PPIs users compared to non-users is 1.89. Cunningham *et al.* (2003) reported a more than two-fold increase in the odds of contracting *Clostridium difficile* colitis in the same patients' population. Risks also take a form of malabsorption of certain food nutrients with their consequent deficiency especially iron and vitamin B12 (Kroupa and Dolina, 2010). In two recent case-control studies (Yang *et al.,* 2006; Targownik *et al.,* 2010), the odds ratio of osteoporosis-related fractures were 1.44 to 2.65 in patients receiving PPIs compared to those who are not. Concerns that therapy with PPIs may mask signs of gastric cancer or even induce precursors of intestinal metaplasia have also been raised. However, unlike animal studies, this has not been fully substantiated in humans (Wayman *et al.,* 1998; Lodato *et al.,* 2010).

Thrombocytopenia is not listed as one of the main side effects of PPIs therapy. However, there have been documented clinical cases of thrombocytopenia with the use of PPIs in patients with peptic ulcer disease (Dotan *et al.,* 2007). In this study, Dotan *et al.* examined the platelet counts of 468 hospitalized patients who were 18 - 80 years of age, prescribed pantoprazole for a minimum of 3 days and were matched to 468 non-medicated controls. The primary outcome was defined as either a drop in the platelet count by >/= 50% relative to baseline, or a drop to < 150,000/ml. Results showed no difference in the occurrence of thrombocytopenia between the two groups, however, a post-hoc analysis revealed a higher incidence of > 20% drop in platelet count in the study group compared with the controls. On the other hand, this adverse effect was not examined previously in experimental animals. Therefore, the current experiment was conducted to explore the effect of chronic administration of one of the PPIs, rabeprazole, on the complete blood count focusing on the platelet count in mice. Monitoring of this side effect may lead to better screening of adverse drug reactions of the PPIs group.

2. Materials and Methods

Experimental animals

All experiments were carried out using male Swiss albino mice weighing 20–25 g. Mice were supplied by the Modern Veterinary Office for Laboratory Animals (Cairo, Egypt) and were allowed to acclimatize for 10 days before starting the experiment. Mice were housed in stainless steel cages in a normal light–dark cycle at around 25 ± 4 °C. Animals were allowed free access to standard diet and water *ad libitum*. Cage substrate (wood shavings) was changed twice weekly with food and tap water *ad libitum*.

Experimental design

Forty mice were randomly allocated into two groups, twenty mice each.

Group I: control group, received distilled water (0.2 ml/mouse/day, p.o.) for six months.

Group II: mice treated with rabeprazole (10 mg/kg/48 h, p.o.) for six months in a volume eq. 0.2 ml/mouse.

In general, rabeprazole sodium (Sigma Pharmaceutical Company, Quesna, Egypt) was dissolved in distilled water and it was given by gastric lavage for a period of six months.

Hematological analysis

Blood was obtained from the retro-orbital plexus using an untreated capillary tube (Fisher, St. Louis, MO). A fresh sample was collected by direct dripping into Microtainer Brand Tubes with EDTA (Becton-Dickinson, Franklin, NJ) as an anticoagulant and used for complete blood count (CBC). Samples were sent for analysis on ice packs and analyzed within 2 h in an automated cell counter (Cell-DYN 1700, Model: CD-1700, ABOTT Diagnostics, USA). The hematological analysis included a complete blood count (CBC). A CBC is a panel of tests that evaluates the three types of cells circulating in the blood and includes the following:

1) Evaluation of white blood cells, the cells that are part of the defense system against infections and cancer and also play a role in allergies and inflammation:

- **White blood cell (WBC) count:** is a count of the total number of white blood cells in a sample of blood.
- **White blood cell differential:** identifies and counts the number of the various types of white blood cells present. These types include neutrophils, lymphocytes, monocytes, eosinophils, and basophils. These cells are present in the blood at relatively stable numbers. The numbers may temporarily shift higher or lower depending on what is going on in the body. For instance, an infection can stimulate the body to produce a higher number of neutrophils to fight off bacterial infection. With allergies, there may be an increased

number of eosinophils. An increased number of lymphocytes may be produced with a viral infection. In certain disease states, such as leukemia, abnormal (immature or mature) white cells rapidly multiply, increasing the WBC count.

2) Evaluation of red blood cells, the cells that transport oxygen throughout the body. They are produced in the bone marrow and released into the bloodstream as they mature. RBCs normally are uniform with minimal variations in size and shape; however, significant variations can occur with conditions such as vitamin B12 and folate deficiencies, iron deficiency, and with a variety of other conditions.

- Red blood cell (RBC) count: is a count of the actual number of red blood cells in a sample of blood.
- Hemoglobin: measures the amount of the oxygen-carrying protein in the blood.
- Hematocrit: measures the percentage of a person's blood that consists of red blood cells.
- Red blood cell indices: are calculations that provide information on the physical characteristics of the RBCs and include:

  - Mean corpuscular volume (MCV): is a measurement of the average size of RBCs and it is calculated by using the following formula: MCV, expressed in femtoliters (fl, or 10-15L)

$$MCV = \frac{\text{hematocrit}(\%) \times 10}{\text{RBC count}(\text{millions} / \text{mm}^3 \text{ blood})}$$

  - Mean corpuscular hemoglobin (MCH): is a calculation of the average amount of oxygen-carrying hemoglobin inside a red blood cell and it is calculated by using the following formula:
    MCH = (Hgb in gmx10)/RBC in millions

  - Mean corpuscular hemoglobin concentration (MCHC): is a calculation of the average percentage of hemoglobin inside a red cell and it is calculated by using the following formula:

$$MCHC = \frac{\text{hemoglobin}(g / 100ml) \times 100}{\text{hematocrit}(\%)}$$

  - Red cell distribution width (RDW): is a calculation of the variation in the size of RBCs and it is calculated by using the following formula:

RDW = (Standard deviation of MCV ÷ mean MCV) × 100

3) Evaluation of platelets (thrombocytes), cell fragments that are vital for normal blood clotting: a decline in the number of blood platelets leads to an increased risk of excessive bleeding and bruising

- The platelet count: is the number of platelets in sample of blood.
- Mean platelet volume (MPV): is a calculation of the average size of platelets. A high MPV indicates increased production of platelets, and a low MPV, indicates decreased production of platelets. It is calculated by using the following formula:

$$MPV\ (fl) = \frac{\text{Thrombocytocrit}}{\text{Thrombocyte count}} * 10$$

Where Thrombocytocrit (Tct) = PDW x thrombocyte count

- Platelet distribution width (PDW): is a measurement of the variation of platelet size.

Statistical analysis

Results were collected, tabulated and presented as mean ± SEM. Statistical analysis was performed using unpaired Student's t-test. The Statistical Package of Social Science (SPSS) program version 17, (Chicago, IL, USA) was used for the statistical analysis. The differences were considered significant at $P < 0.05$.

3. Results

In the present study, a complete blood count was performed after chronic treatment with rabeprazole sodium in mice. Student's t-test revealed that there was no significant difference in the RBCs count in rabeprazole-treated mice compared to normal mice (8.59±0.18 vs. 7.92±0.2, $P < 0.05$, Table 1). Similarly, blood hemoglobin and hematocrit % were not different among the two groups.

Hematological parameters related to RBCs showed that the MCV value, MCH, MCHC and RDW % were not different in the current two experimental groups (38.17 ± 0.4 vs. 38 ± 0.05; 14.47 ± 0.27 vs. 13.9 ± 0.01; 37.93 ± 0.83 vs. 36.65 ± 0.05; 12.55 ± 0.14 vs. 12.3 ± 0.4, respectively, $P < 0.05$, Table 1).

Importantly, measuring the platelet count demonstrated that chronic treatment with rabeprazole sodium produced thrombocytopenia; there was a significant drop in platelet count from 1783 ± 65 in the normal mice to 1345.6 ± 103.75 in rabeprazole-treated mice ($P < 0.05$, Table 1). MPV value was not different between the two groups. The same way Pct % and PDW % were not different between the two groups ($P < 0.05$, Table 1)

The results of the present study indicated that chronic rabeprazole treatment produced leukopenia in mice. There was a decline in the total count of white blood cells from 9.9 ± 0.4 in the

normal mice to 7.03 ± 0.76 in rabeprazole-treated mice ($P < 0.05$, Table 2). Differential leukocyte count revealed non-significant declines in the lymphocytes, monocytes, neutrophils, eosinophils, basophils percent between the two groups indicating that leukocytopenia was unrelated to deficiency in a certain population of these white blood cells but was almost related to a decline in all white blood cell types (Table 2).

Table 1: Effect of rabeprazole (10 mg/kg/day) on complete blood count in mice

Parameters	Normal	Rabeprazole sodium (10 mg/kg/day)
RBC (M/µL)	7.92 ± 0.2	8.59 ± 0.18
Hgb (g/dl)	10 ± 0.2	10.92 ± 0.3
Hct (%)	30 ± 0.7	32.77 ± 0.88
MCV (fL)	38 ± 0.05	38.17 ± 0.4
MCH (Pg)	13.9 ± 0.01	14.47 ± 0.27
MCHC (g/dl)	36.65 ± 0.05	37.93 ± 0.83
RDW (%)	12.3 ± 0.4	12.55 ± 0.14
Platelet (K/µL)	1783 ± 65	1345.6 ± 103.75[a]
MPV (fL)	16.95 ± 1.65	15.72 ± 0.51
Pct (%)	0.5 ± 0.01	0.5 ± 0.01
PDW (%)	0	3.87 [a]

RBCs: red blood cells, Hgb: hemoglobin, Hct: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width, MPV: mean platelet volume, Pct: plateletcrit, PDW: platelet distribution width. Mice were treated with rabeprazole-sodium for six months. Results are expressed as mean ± S.E.M. and analyzed using unpaired student's t test at $P < 0.05$. [a] Compared to normal group, $n = 20$.

Table 2: Effect of rabeprazole (10 mg/kg/day) on leukocyte formula in mice.

	Normal	Rabeprazole sodium (10 mg/kg/day)
WBC (K/µL)	9.9 ± 0.4	7.03 ± 0.76[a]
Lymphocytes (%)	92 ± 2.12	93.5 ± 1.31
Monocytes (%)	2 ± 0.23	1.83 ± 0.31
Neutrophils (%)	4.5 ± 1.38	3.5 ± 1.02
Eosinophils (%)	1.5 ± 0.14	1 ± 0.37
Basophils (%)	0	0

WBC: white blood cells. Mice were treated with rabeprazole-sodium for six months. Results are expressed as mean ± S.E.M. and analyzed using unpaired student's t test at $P < 0.05$. [a] Compared to normal group, $n = 20$.

4. Discussion

Since the introduction of the first proton pump inhibitor (PPI) in the late 1980's, the use of PPIs has increased exponentially with sharp increases of up to 456% in the 1990's relative to earlier years (Guda *et al* ., 2004). PPIs remain one of the world's most frequently prescribed medications (Garner *et al.*, 1996); concurrently, several studies in the United States and Europe continue to report PPIs overuse in the hospital and ambulatory settings. Furthermore, once PPIs are started in-hospital, previous reports show that more than 50% of patients continue to take them 3 to 6 months after discharge (Nardino *et al.*, 2000; Parente *et al.*, 2003). Although PPIs are generally perceived as safe medications, several reports in the medical literature, mostly case control studies and large population based reports, continue to debate their possible side effects (Eid *et al.*, 2010). The current study focused on a rarely reported adverse effect consists of thrombocytopenia associated with the use of PPIs.

Our data showed that chronic treatment with rabeprazole sodium resulted in a significant drop in platelet count. In accordance with our observation, Zlabek and Anderson (2002) and Watson *et al.* (2006) described cases of thrombocytopenia associated with the administration of lansoprazole and pantoprazole respectively. In the first study (Zlabek and Anderson, 2002) described a case for an 85 year-old white man presented with an upper gastrointestinal hemorrhage from a gastric ulcer. His platelet count was normal on admission. He was started on oral lansoprazole 60 mg twice daily and on hospital day 2, his platelet count decreased to 102 x 10^3/mm^3; on hospital day 3, the platelet count was 36 x 10^3/mm^3. Lansoprazole was discontinued, and the platelet count returned to normal. In the second study, Watson *et al.* (2006) reported two cases of thrombocytopenia associated

with pantoprazole treatment. The authors described the course of thrombocytopenia associated with pantoprazole 40 mg in two hospitalized patients. In both cases, thrombocytopenia appeared after the initiation of pantoprazole and rapidly improved after its discontinuation, although complete resolution of thrombocytopenia occurred in only one patient prior to discharge from the hospital. A more recent report evaluated the case of a 45 year-old man who was admitted to the emergency room with upper gastrointestinal hemorrhage. The patient was given intravenous pantoprazole. The platelet count decreased daily during the hospital course for 3 days, until it reached a nadir of 70 x 10^3 /cc. Peripheral smear showed a reduced platelet count with normal morphology. Pantoprazole was discontinued on hospital day 4, and by days 6 and 8, the platelet count had risen to 100 x 10^3 /cc and 220 x 10^3 /cc, respectively. Pantoprazole was switched to oral rabeprazole on hospital day 8. The patient's platelet count remained stable during the hospitalization (Taş., 2013).

The mechanism of drug-induced thrombocytopenia is often poorly understood, however, thrombocytopenia generally can be caused by a variety of reasons. These reasons can be divided into decreased platelet production, increased platelet destruction or consumption, or increased splenic sequestration (capturing of circulating platelets in the spleen) (Levine., 2004). Increase in platelet destruction may result from immunological processes (idiopathic thrombocytopenic purpura) and/or non-immunological processes (disseminated intravascular coagulopathy, thrombotic thrombocytopenic purpura, hemolytic-uremic syndrome, prosthetic valves, infections, wounding due to the abnormal vascular surface, cavernous haemangioma) (Hope *et al.*, 1981; Tomer *et al.*, 1991). On the other hand, decreased platelet production is usually related to a bone marrow problem. Bone marrow may not be able to produce enough platelets due to several reasons, for example aplastic anemia, leukemia, liver cirrhosis and/or vitamin B_{12} deficiency.

Vitamin B_{12} acts as a co-factor during the synthesis phase of the cells in the bone marrow (Whitehead *et al.*, 2003). Hypersegmented neutrophils are seen as an initial finding in peripheral blood when vitamin B_{12} levels are lowered. After then, megaloblastic anemia, leucopenia and thrombocytopenia occurs (Carmel., 2004). Vitamin B_{12} is an essential nutrient that must be acquired from the diet, and it is present in foods bound to protein. It is well established that the presence of gastric acid is needed for the pancreatic proteases to cleave the vitamin B_{12} from the protein, allowing its reassociation with intrinsic factor and eventual absorption in the terminal ileum (Festen., 199; Termanini *et al.*, 1998; Jensen., 2006; Pohl *et al.*, 2008; McColl., 2009). In short-term studies various acid suppressants (H_2RA, PPIs) have been reported to decrease the absorption of vitamin B_{12} from foods, but not to decrease absorption of crystalline vitamin B_{12} which is not protein bound (Steinberg *et al.*, 1980; Dutta., 1994)

In 2008, two studies evaluated the effects of PPIs on vitamin B_{12} status and came to different conclusions. In the first study (Dharmarajan *et al.*, 2008), the effects of chronic use of H_2RA (150 pts), PPIs (141 pts) or neither (251 pts) were examined in elderly patients in nursing homes or the community ambulatory care facilities. In 20% of the nursing home patients and 29% of the community care patients low/marginally low vitamin B_{12} status was found which is consistent with a number of other studies reporting 25% in such patients with a range of 3–40% (Valuck and Ruscin, 2004; Dharmarajan and Norkus, 2008). These results demonstrate that PPIs, but not H_2RA usage was associated with lower vitamin B_{12} levels, the percentage decrease in vitamin B_{12} levels correlated with the time of PPIs usage, and concomitant use of oral vitamin B_{12} did not prevent this decrease, and it only delayed it. In the second study (den Elzen *et al.*, 2008), serum vitamin B_{12} levels were compared in 125 aged [>65 yrs] long term PPIs users and their partners not taking PPIs. No differences in serum vitamin B_{12} levels were detected.

Moreover, in 2010, Rozgony *et al.* examined 34 long-term care patients aged 60-80 years (17 taking long-term PPIs, 19 not taking PPIs) and the effect of a vitamin B_{12} nasal spray for 8 weeks on the vitamin B_{12} status. At baseline the chronic PPI users had lower serum vitamin B_{12} levels, higher methylmalonic acid levels (MMA) and a greater percentage were vitamin B_{12} deficient (75 vs. 11%, p=0.006). After 8 weeks of vitamin B_{12} nasal spray (500mcg/once per week), there was a significant increase in serum vitamin B_{12} levels compared to pretreatment in the chronic PPIs users, and a significant decrease in the frequency of vitamin B_{12} deficiency in the chronic PPIs uses from pretreatment (75 to 24%, p=0.012). These authors conclude that older individuals who are long-term PPI users are at increased risk of vitamin B_{12} deficiency and should be more systematically screened for vitamin B_{12} deficiency than is currently performed in most institutions of chronic care.

All of the above mentioned data may suggest a proposed mechanism for the PPIs induced thrombocytopenia observed in the current work, which is the development of lower vitamin B_{12} levels, and an increased frequency of vitamin B_{12} deficiency. However, this is still not firmly established, is not widely acted on, and thus remains controversial. More

experimental and randomized trials are needed. With the available information, it would seem appropriate to evaluate vitamin B_{12} and platelet status at appropriate intervals in long term users of PPIs.

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