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Research Article

Histologic, Histomorphometric and Acid Secretory Changes Accompanying Gastric Ulcer Healing in Lead-Exposed Rats

Adeleye G.S[1.2] and Olaleye S.B[1*]

Department of Physiology, University of Ibadan, Ibadan. Ibadan, Nigeria.
Department of Physiology, Afe Babalola University, Ado-Ekiti. Ekiti State, Nigeria.

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Abstract

Reported cases of environmental lead exposure and its effects on the health of humans and livestock are on the increase globally. Lead affects many biological systems leading to death if it accumulates beyond certain limits. Previous reports have shown that experimental lead exposure predisposes the stomach to gastric ulceration. However, it is not known if Lead interferes with gastric ulcer healing process. In this study, the effects of chronic lead exposure on healing of gastric ulceration with focus on histologic and gastric acid secretory changes were investigated. Sixty rats (80-100 g) were randomly divided equally into control (tap water), low and high dose (Lead acetate, 100 ppm and 5,000 ppm respectively, p.o.) groups. Twenty weeks post-treatment, ulcers were induced in the stomachs of the animals by serosal application of 60% acetic acid after anesthesia (thiopental sodium: 50 mg/Kg). In the control animals, the ulcer area was 7.9±0.1mm² by day 7 and 4.8±0.1 mm² by day 14. Lead exposure significantly increased ulcer area for low (10.0±0.4mm² and 8.0±0.4mm²) and high (14.4±0.2mm² and 10.5±0.3mm²) doses respectively. Histomorphometry revealed increased ulcer width (120.8%) in the high lead group when compared with control (100%) on day 14. Histology revealed gastric ulceration in all groups on day 7 with presence of inflammatory cells at ulcer bed. By day 14, marked reduction in the inflammatory cells and greater fibroblast proliferation were observed in control compared with the treated groups. Basal acid secretion reduced over the healing period at a lower rate in the treated groups (30.4% for low, 40.0% for high lead groups) when compared with control (60.5%). Compared with control (44.2±2.0 and 55.2±1.8/unit area on days 7 and 14), lead exposure decreased Goblet Cell Count during healing in treated groups (32.8±0.9 and 33.2±0.9 for low lead and 33.8±1.2 and 35.6±1.2 for high lead) for the same days.

Keyword: Lead, Ulcer healing, histomorphometry, gastric acid, histology, rats

INTRODUCTION

The gastrointestinal tract (GIT) is a very important system in the body, being the only natural route through which nutrients and other biological and non-biological molecules (condiments, drugs, e.t.c) needed for growth and development are ingested. All substances needed by living organisms for survival enter the stomach from the mouth where they are digested and/or absorbed. One of the most important secretions of the stomach that aids digestion and absorption of food is gastric acid. Alterations in gastric acid secretion has been implicated in some diseases with excessive secretion leading to gastritis and peptic ulcer disease (Peek and Blasser, 1997).

Peptic ulcer is a common disease throughout the world and in the recent past, there has been a huge increase in contribution to the knowledge on the treatment of the disease (Ige *et al*, 2010). Peptic ulcer is defined as integrity disturbance of gastric and/or duodenal mucosa which causes local defect or excavation due to active inflammation (*Syam et al 2007*). It is a major health problem with multifactorial aetiology (Ajaiyi *et al*, 2012). The process of digestion and absorption makes the stomach to be susceptible to normal "wear and tear". However, there are some mechanisms found in the stomach that protect it from these assaults and minimize the damage that would otherwise have occurred. The development of gastric ulcer subsequent to acidic digestion of the mucosal defense (Ajaiyi *et al*, 2012).

Although not hitherto given much attention, it is becoming clear that environmental toxicants (e.g. heavy metals) are fast being recognized as major sources of assault on the gastrointestinal tract (and the stomach in particular). Examples of some heavy metals that are found in the environment are lead, cadmium, mercury, chromium, arsenic, vanadium etc. Lead is a ubiquitous and versatile metal which has been used by mankind for many centuries. It ranks as one of the most serious environmental toxicants amongst the toxic heavy metals all over the world. Mankind has used it for many years because of its wide variety of applications. Human exposure to lead is from numerous sources and a myriad of pathways including air, food, dust, soil, work place and water (Menezes, D'souza, and Venkatesh, 2003; Herman, Geraldine and Venkatesh, 2007). Lead poisoning is a medical condition caused by increased levels of lead in the body. Symptoms of lead poisoning (in the gastrointestinal tract) include abdominal pain constipation, colic, diarrhea, poor appetite, etc.

**Author for Correspondience:* .: +234-8023255893
E-mail: sbolaleye@yahoo.com

Environmental lead contamination is present in both industrialized and rural societies and poses significant health hazards (Alperstein *et al*, 1991).

In previous studies, it was shown that exposure of laboratory animals to lead enhanced the formation of experimentally induced ulcers (Olaleye *et al* 2006). This has been attributed to increased oxidative stress in the gastric mucosa of exposed rats (Olaleye *et al*, 2007). Vahedian *et al.*, (2011) also stated that lead exposure causes increased nitric oxide (NO) levels in stomach tissue of rats. The increased NO levels correlated with increased acid output in exposed rats, the increased acid output delays ulcer healing by inhibiting cell migration and maturation of the granulation tissue (Schmassmann 2010). However, whether exposure to Lead will affect the processes leading to healing of such ulcers in the stomach is not known. Unlike gastroprotection which depends on the balance between defensive (mucous, bicarbonate, etc) and aggressive (acid, pepsin, h. pylori, etc) factors, healing of ulcers involves processes such as granulation tissue formation, re-epithelialization, tissue remodeling, cell proliferation, cell division, cell hypertrophy, clot formation, phagocytosis, fibroblast migration, collagen deposition, vascular remodeling, mucosal regeneration, reconstruction of gastric glands, angiogenesis, muscle restoration, etc (Tarnawski, 2000).

The present study was aimed at studying the effects of lead exposure on the rate of healing of acetic acid-induced ulceration, with special interest on gastric histology and acid secretion.

MATERIAL AND METHODS

Animals: Sixty (60) healthy male albino rats of Wistar strain weighing between 80-100 grams were used for the study. These animals were divided into 3 groups of 20 rats each. The animals were housed under standard conditions of temperature (23±2ºC), humidity (55±15%) and 12hr light/dark cycles (7.00am-7.00pm) in the Animal house of Department of Physiology, University of Ibadan. They were kept in wire meshed cages with beddings which were adequately changed throughout the period of study. Animals were allowed to acclimatize to animal house conditions for two weeks with free access to commercial rat chow and tap water *ad libitum* before commencement of studies.

Chemicals and Reagents: Some of the chemicals and reagents used in this study are: Lead Acetate (99%, BDH Chemicals Ltd Poole, England), Concentrated Acetic Acid (99%), Sodium Chloride, Thiopental Sodium (Rotex Medica, Germany). Histamine and other analytical grade chemicals used were obtained from British Drug Houses, UK.

Animal grouping: The rats were randomly divided into three groups: control (drank tap water for 20 weeks), low-dose (100 parts per million (ppm) of Lead Acetate dissolved in drinking water for 20 weeks) and high-dose (5,000 ppm of Lead Acetate dissolved in drinking water for 20 weeks) groups. This method of lead exposure has been used previously by Delville (1999). Each group has twenty animals distributed evenly among four subgroups. The four subgroups were animals sacrificed on days 0 (6 hours after ulcer was induced), 7, 14 and 21 respectively after ulcer induction in all groups (Gruber *et al*, 1997).

Induction of Ulcer using Acetic acid: At the end of 20 weeks, ulcer was induced by the method of Wang *et al* (1989) with slight modifications. Food was withheld 24-36 hrs before ulcer induction, while having access to water. The animals were anaesthesized with Thiopental Sodium (50mg/Kg) and laparatomy was performed to expose the stomach. Acetic acid (0.5ml, 60% vol/vol) was applied to the serosal surface of the glandular portion of the stomach for 1 minute using a 3ml syringe barrel which had been cut and smoothed. The acid was removed by aspiration and the area washed with sterile saline and dabbed with cotton wool. The abdomen was sutured and the animals returned to their diets and water. The animals were sacrificed at intervals to check for ulcer healing (i.e. on days 0 (6 hours after ulcer was induced), 7, 14, and 21 post-ulcer induction).

Histomorphometry: After six hours as well as on days 7, 14 and 21 post ulcer induction, five animals were randomly picked from each group, sacrificed by cervical dislocation, their stomachs removed and opened along greater curvature and then rinsed with normal saline. Histomorphometric analysis was carried out according to the method described by Ofusori *et al.*, (2008) and reported earlier by Ajayi and Olaleye (2015). Briefly. the eyepiece of the occulometer was divided into two 100 small divisions (the stage micrometer scale was made up to 1mm divided into 0.1mm divisions and each 0.1mm was divided into 0.01mm, the eye piece scale and then inserted into the eye piece of the microscope by removing the superior lens thus placing the scale on the field stop, the stage micrometer was also placed on the stage of the microscope, the stage scale was focused by the low power objective lens (x4), the stage and the eye piece scales were adjusted until there was a parallel point between the two scales, the number of the eye piece divisions and its corresponding stage measurements was noted; (if 70 occulometer divisions equal to 14µm, all the objective lens were thus calibrated). Using the histological slides previously prepared, fibroblast, parietal and mucous cells numbers and dimensions were estimated under the microscope.

Histological studies*:* To process for histopathological studies, gastric specimens were fixed in 10% formalin in phosphate buffered saline, embedded in paraffin and cut into 4 micrometer sections. Paraffin sections were deparrafinized with xylene, hydrated and stained with haematoxylin and eosin. The stained sections were assessed for any inflammatory/other pathologic changes including infiltration of cells, necrosis or damage to nucleus or tissue structures. Histomorphometric studies were done at the Department of Veterinary Anatomy, University of Ibadan. A graticle with 1-100µm calibration was attached to the microscope. Using the histological slides previously prepared, fibroblast, parietal and mucous cells numbers and dimensions were estimated under the microscope. Also, ulcer depth and width were measured and recorded, the mucosal area eroded was then calculated.

Assay for Plasma Lead levels: Plasma lead levels in all groups was assayed using spectrophotometric techniques at the Multidisciplinary Central Research Laboratories, University of Ibadan, Ibadan, Nigeria.

Gastric acid secretion: Gastric acid secretion was studied in normal rats and those with acetic acid induced ulcers using the

continuous perfusion technique described originally by Ghosh and Schild (1958) via a modified Langerdoff perfusion apparatus. After confirming anesthesia (xylazine/ketamine mixture), A small cut was made in the upper part of the trachea and a 2.5cm long polythene size 3 cannula was inserted into it. The thread below the trachea was then tied lightly round the inserted cannula. The significance of trachea cannulation was to avoid any respiratory difficulties, by-passing the nasal passage to the larynx. Also with the cannula in place, any fluid accumulating in the trachea can be readily aspirated. Also the essence of exposing the oesophagus before cannulating the trachea was to prevent puncturing the esophagus during the passage of esophageal cannula from the mouth. To prevent drying up of the exposed tissue, saline soaked cotton wool was placed over the dissected area. Another size 3 polythene cannula from a Lagendorff apparatus was passed into the oesophagus to reach the cardia region of the stomach. The linear alba was cleared of hair, a midline incision was made through the skin and muscle of the abdomen. The stomach was brought out, a small cut was made about an inch distal to the pyloro-duodenal junction, the perfusion fluid maintained at 37 ^{o}C was rushed from the Lagendorff apparatus tap to wash out all food debris from the stomach of the animal. With the perfusion fluid still running, the early part of the duodenum was cannulated and the stomach was packed back into the peritoneum. The muscle was sutured back before the skin. It was ensured that the perfusion fluid was running out of the stomach. After collecting consistent basal output, 0.5mg/kg histamine acid phosphate was injected into each animal intramuscularly

The rate of flow of the perfusing fluid was manually adjusted such that 10 mL of effluent (which contains gastric juice from the stomach) was collected at 10 minute intervals. The first few effluents (4-5) were collected to establish a consistent basal output. After each sample collection, 5 mL of the 10mL collected was measured into a conical flask and two drops of phenolphthalein reagent was added. 0.0025M NaOH was run dropwise into the cornical flask, shaking the flask in the process until the first appearance of a pink colour. After this, the titration was stopped and the end point recorded

Statistical Analysis

The data obtained were expressed as mean and standard error of mean (Mean ± SEM) wherever possible. The difference between the means was determined using independent sample Students t-test. P values less than or equal to 0.05 were considered significant using Student t-test.

RESULTS

Plasma lead levels of rats after twenty weeks of chronic lead exposure

As shown in Fig. 1, plasma lead level in the control (unexposed rats) was 0.0102±0.0008µg/L. In the animals exposed to 100ppm and 5000ppm lead, plasma levels of lead significantly increased to 0.564±0.0254µg/L ($p<0.001$) and 0.992±0.0525µg/L ($P<0.0001$) respectively when compared with the control.

Effect of chronic lead exposure on ulcer healing rates

Administration of acetic acid produced gastric ulcers in all animals (severity of 100 percent).

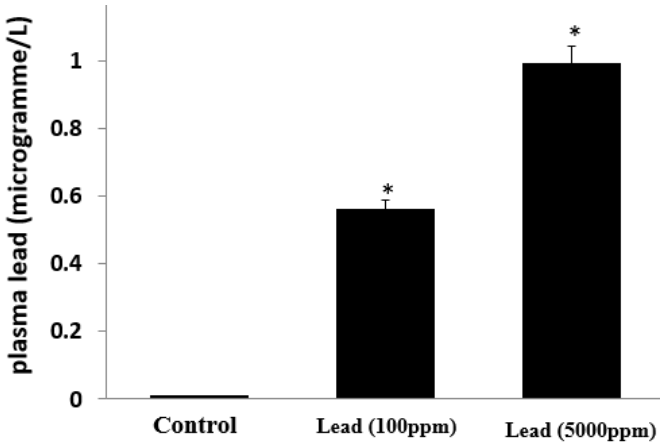

Figure 1:
Effects of chronic lead exposure on plasma lead levels in rats after twenty weeks' exposure (*$p<0.0001$)

Figure 2 shows the effect of chronic lead administration on the rate of ulcer healing. By day 7 post ulcer induction, the ulcer area in the control group was 7.90±0.12µm^2. In the low and high lead exposed groups, the ulcer areas on day 7 were 9.98±0.41µm^2 and 14.36±0.21µm^2 respectively. As healing progressed significant reductions in ulcer areas were observed in all the stomach samples. However, there were significant differences in the rate of decline in the ulcer areas. In the control rat stomach, ulcer area reduced significantly from 7.90±0.12µm^2 to 4.82±0.14µm^2 on day 14 (38.99% healing). In the 100ppm lead treated group, ulcer area decreased from 9.98±0.41µm^2 on day 7 to 7.98±0.36µm^2 on day 14 (20.04% healing), while in the 5000ppm lead treated group, ulcer area decreased from 14.36±0.21µm^2 on day 7 to 10.46±0.32µm^2 on day 14 (27.16% healing). By day 21, the ulcer healing rates were 69.71%, 63.41% and 62.91% respectively for control,

Effects of chronic lead exposure on gastric ulcer width and mucosal area eroded: Figure 3 shows the effects of chronic lead exposure on gastric ulcer width in control and lead treated rats. On day 7, the ulcer width in the low lead (low dose) group was 654.9±12.2 µm, and high dose group was 601.2±38.2 µm, compared with control value of 550.5±17.8 µm. On day 14, the ulcer width in the low dose and high dose were 193.20±7.69 µm and 180.53±14.11 µm compared to control value of 149.1±3.7µm. Finally, on day 21, the ulcer width in the low dose and high dose groups were 71.1±3.1 µm and 109.5±9.2 µm relative to control value of 52.8 µm All differences were statistically significant ($P<0.05$). Ulcer width and mucosal areas eroded (on days 7, 14 and 21) in treated animals were higher than control animals indicating a slower rate of ulcer healing in the treated animals

On day 7, the gastric mucosal area eroded in the low dose group was increased by 190.5% of the control value while the ulcer width of the high dose group increased by 208.6% on the same day compared to control value of 100% (Table 1). On day 14, the mucosal area eroded in the low lead (low dose) group was increased by 196.1% of the control value while the ulcer width of the high dose group increased by 244.3% on the same day. Finally, on day 21, the mucosal area eroded in the low lead (low dose) group was increased by 151.1% of the control value while the ulcer width of the high dose group increased by 417.6% on the same day relative to control. All differences were statistically significant ($P<0.05$).

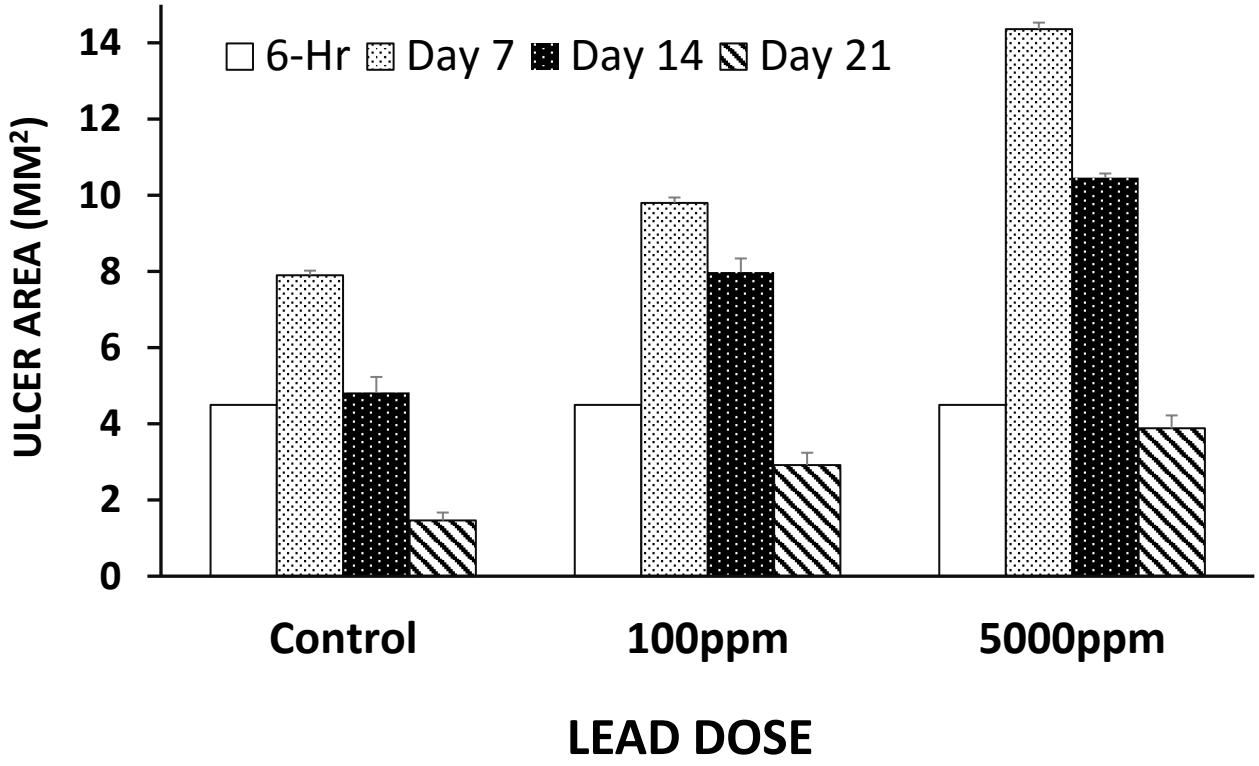

Figure 2
Effect of chronic lead treatment on ulcer areas on 6hours and days 7, 14 and 21 after ulcer induction by acetic acid

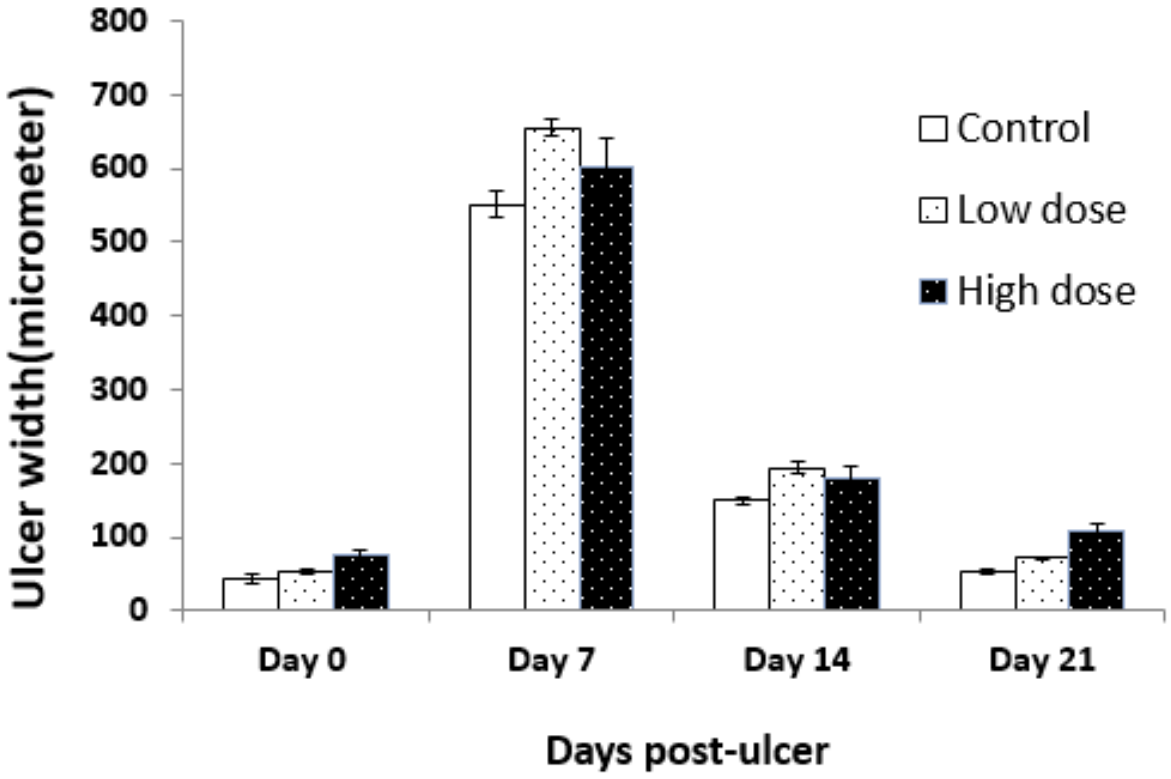

Figure 3:
Effects of chronic lead exposure on gastric ulcer width after ulcer induction (Day 0 represents data taken six hours post ulcer induction). Each bar represents mean±SEM of n)

Table 1:
Effects of chronic lead exposure on gastric mucosal area eroded after ulcer induction (histomorphometry)

Groups	Day 0 (µm²)	Day 7(µm²)	Day 14(µm²)	Day 21(µm²)
Control	10200.00±44.04	25800.00±121.38	46100.00±25.34	13100.00±27.4
Low dose	16200.00 ±16.29	49100.00±83.25*	90400.00±52.54*	19800.00±21.08
High dose	39900.00 ±52.67*	538100.00±26.09*	112000.00±96.4*	54700.0062.5*

*= Significant difference at P<0.05

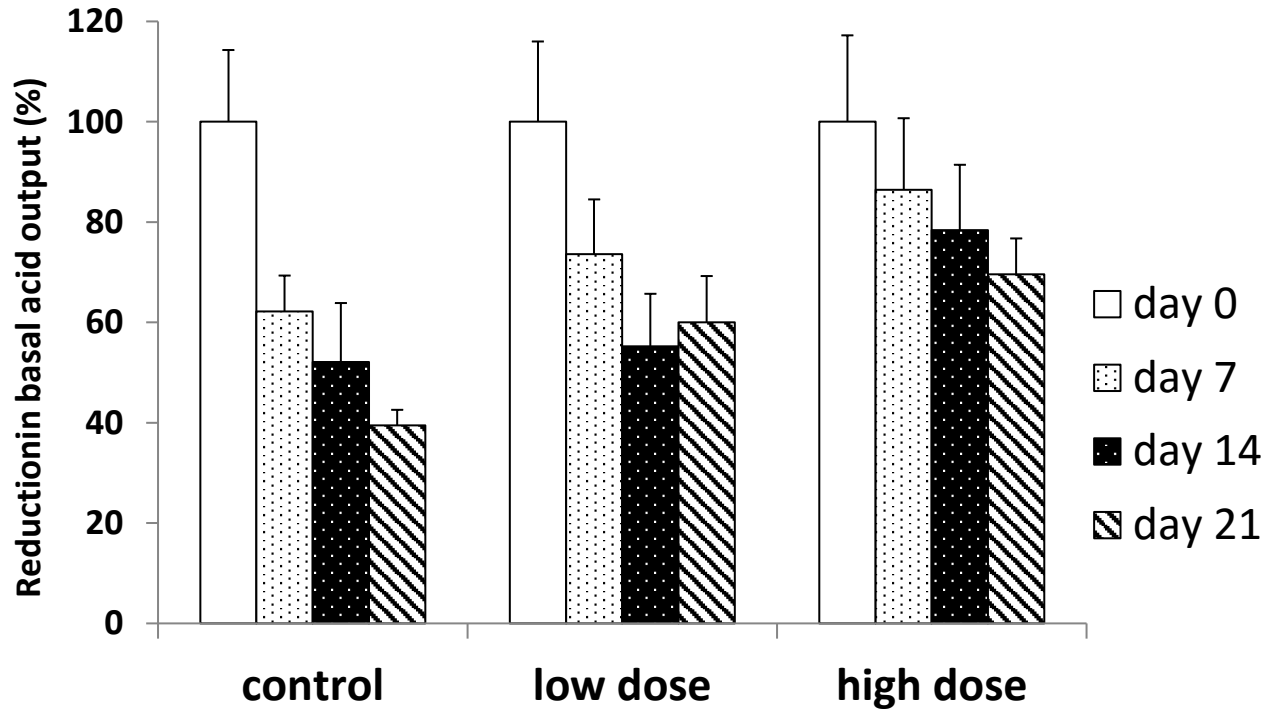

Figure 4
Effect of chronic lead treatment on Gastric acid secretory changes 6hours and days 7, 14 and 21 after ulcer induction by acetic acid

Effect of chronic lead treatment Gastric acid secretion during healing of acetic acid induced ulcers: Figure 4 shows Basal Gastric Acid Secretion (BGAS) changes in control and lead-treated rats after acetic acid induced ulceration. Six hours after ulcer induction, there was no significant difference between BGAS of the treatment groups and control. As healing progressed (days 7-21) the BGAS decreased progressively in all groups. In the control group, BGAS on day 0 was 2.38±0.34 mEq/L. This was decreased to 1.48± 0.17 (62.2%), to 1.24 ± 0.28 (52.1%) and 0.94±0.07 (39.5%) on days 7, 14 and 21 respectively. In the low lead treated animals, BGAS was 2.5± 0.38 mEq/L on the day of ulcer induction. However, BGAS decreased to 1.84± 0.26 (73.6%), and to1.38± 0.25 (55.2%) and 1.50±0.22 (60%) on days 7, 14 and 21 respectively. In the high lead treated group, BGAS on the day of ulcer induction was 2.50± 0.41mEq/L. This was decreased to 2.16 ± 0.34 (86.4%) on day 7, 1.96 ± 0.31 (78.4%) on day 14 and 1.74±0.17 (69.6%) on day 21 respectively. It was noted that at the end of the experiment, control animals had the lowest absolute BGAS and the difference was significant when compared to high dose group.

Figure 5 shows the peak histamine-stimulated gastric acid (PHSGA) output and the percentage changes in PHSGA over basal in control and lead-treated rats after acetic acid induced ulceration. Six hours after ulcer induction, there were no significant differences between the PHSGA of the control and treatment groups (P<0.05). As healing progressed (days 7-21), PHSGA decreased progressively in all groups. In the control group, PHSGA secretion on day 0 was 5.18±0.41mEq/L. This was decreased to 4.24±0.20 mEq/L (81.9%), and to 3.52±0.37mEq/L (68.0%) and 2.50±0.12mEq/L (48.3%) on days 7, 14 and 21 respectively. In the low lead treated animals, PHSGA secretion was 5.17±0.46 mEq/L on the day of ulcer induction. However, PHSGA secretion decreased to 4.22±0.34 mEq/L (81.6 %), and to 3.58±0.53 (69.2 %) and 3.70±0.48 (71.6 %) on days 7, 14 and 21 respectively. In the high lead treated group, PHSGA output on the day of ulcer induction was 5.22±0.33 mEq/L. This was decreased to 4.84±0.39 mEq/L (92.7%) on day 7, 4.44±0.23 mEq/L (85.1%) on day 14 and 3.92±0.28mEq/L (75.1%) on day 21 respectively. Note that at the end of the experiment, control animals had the lowest absolute PHSGA output and the difference was significant when compared to both low and high dose groups.

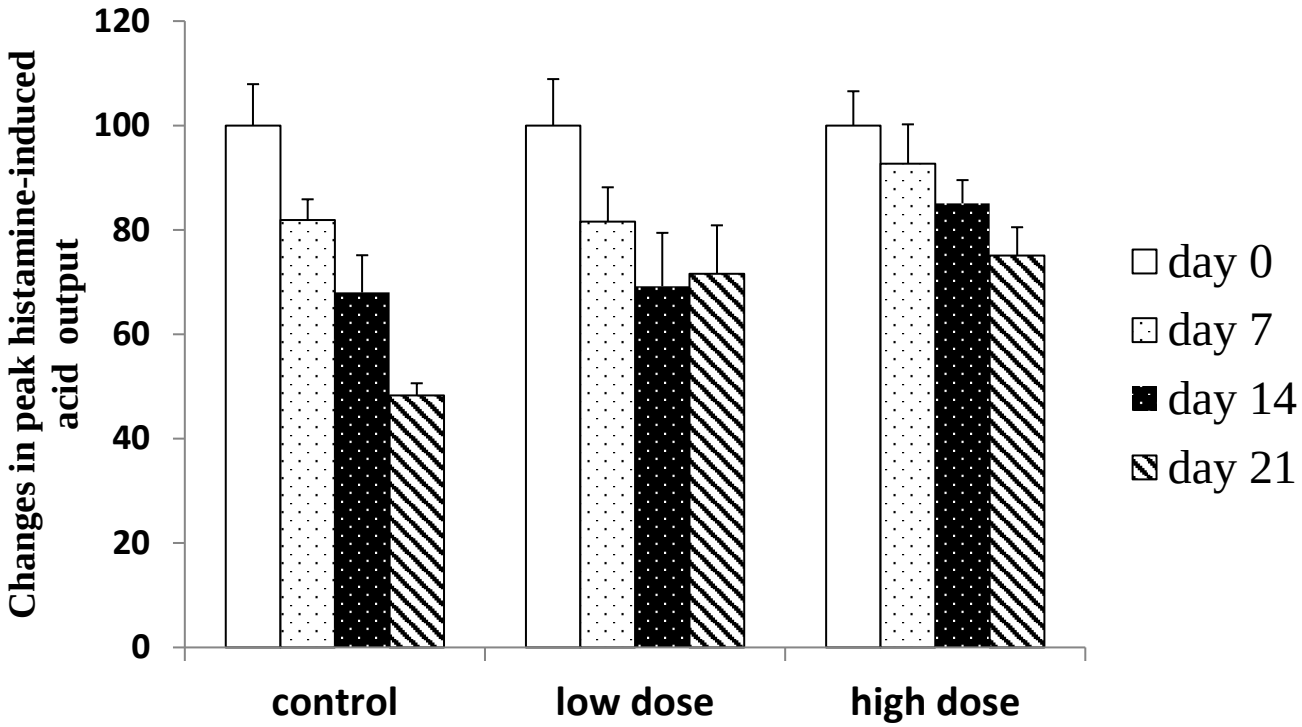

Figure 4
Effect of chronic lead treatment on peak histamine-stimulated gastric acid output 6hours and days 7, 14 and 21 after ulcer induction by acetic acid

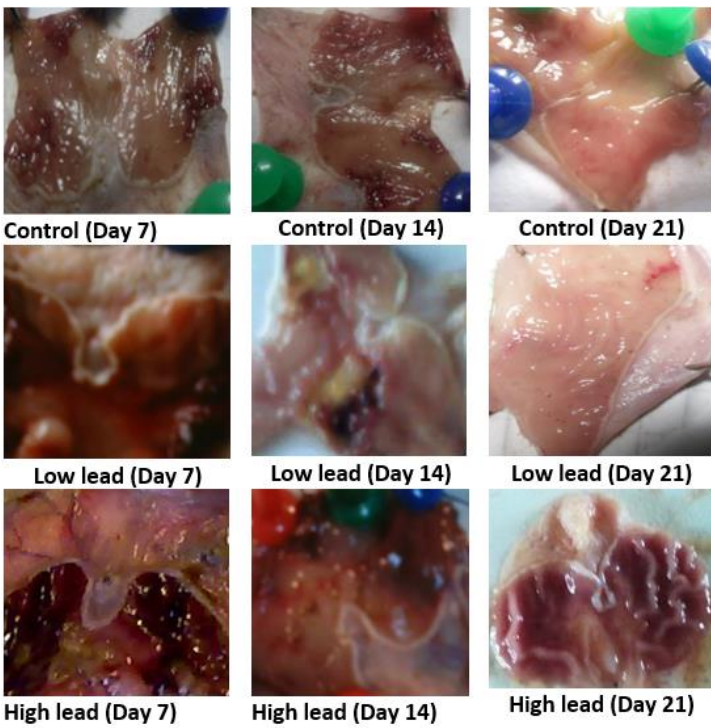

Plate 1:
Gross morphological appearance of stomach samples from rats exposed to lead for twenty weeks prior to induction of ulcer by acetic acid.

Changes in Goblet Cell Counts (GCC) after acetic acid induced ulcerated in control and lead treated rats

As shown in Table 2, six hours after ulcer induction, GCC in both treated groups were 25.4 and 22.2 cells/unit area (for low and high doses respectively) compared to control value of 57.0 cells/unit area. The differences were significant ($P<0.01$). In the control animals during healing, goblet cell count increased from 44.2 on day 7 to 55.2 on day 14, but by day 21, it reduced to 34.2 cells /unit area.

During healing (days 7-21), in both treated groups, there were insignificant changes in goblet cell counts as healing progressed (days 7-21). The goblet cell counts were significantly lower in the treatment groups compared to control ($P<0.05$) during healing except in day 21 which was not significant.

DISCUSSION

The results of this study show a significant difference in blood lead levels in both treated groups when compared with the control. Similar observations have been made in previous reports by Olaleye *et al*., (2006; 2007) and Vahedian, (2011) where lead exposed animals had higher plasma lead levels than control. It also confirms the efficacy of the method of lead exposure via drinking water.

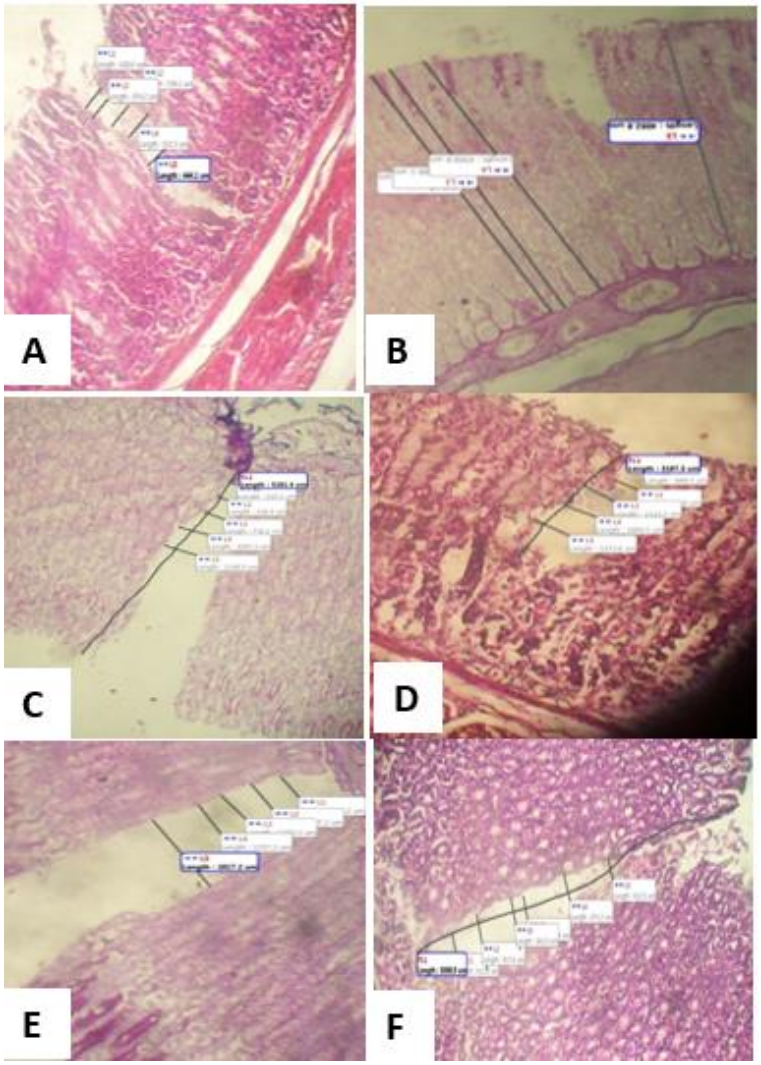

Plate 2:
Gastric mucosa sections showing ulcer width and depth in control rats on (A) day 7 and (B) day 14 after acetic acid induced ulceration. Note the reduced ulcer depth on day 14 compared to day 7. C and D are representative slides from rats treated with 100ppm lead and thereafter with acetic acid induced ulcers. Note the deep defect caused by the acetic acid (C) and partial filling of defect by day 14 (D). Slides E and F show width and depth in stomach of high lead exposed rats on days 7 and 14 respectively. Note the deep defect caused by the acetic acid and partial filling of defect by day 14; note also the narrowing of the ulcerated area on day (H&E X100)

Table 2:
Effects of chronic lead exposure on goblet cell count after ulcer induction

Groups	Day 0	Day 7	Day 14	Day 21
Control	57.0 ±1.64	44.2 ±2.03	55.2 ±1.77*	34.2 ±1.65#
Low dose	25.4 ±1.21	32.8 ±0.86	33.2± 0.86NS	31.0± 0.45NS
High dose	22.2 ±0.86	33.8 ±1.16	32.2± 1.02NS	35.6 ±1.21NS

Values are presented as mean ± SEM, N = 5,
*=significant compared with animals in same group on day 7 $p < 0.05$
#=significant compared with animals in same group on day 14 $p<0.05$
NS= not significant compared with the previous day in same group

Healing of ulcers is a regular, physiological process aimed at restoring damaged area to normal state. In the gastrointestinal tract, healing involves a well-orchestrated sequence of events – hemostasis, inflammation, cell proliferation, re-epithelialization, formation of granulation tissue, angiogenesis, interactions between various cells and the matrix and tissue remodeling (Tanawski, 2005; Guo and DiPietro, 2010). At the molecular level, several factors (growth factors, cytokine, transcription factors, etc) have been shown to affect these processes. However, at the tissue level, it has been shown that for healing to be fully accomplished, some processes must occur. In the present study, we investigated the effects of chronic lead treatment on gastric acid secretion as well as macroscopic and microscopic changes as healing progressed in the rat stomach. In previous studies, it was reported that exposure of rats to lead increased the incidence of ulcer formation (Olaleye *et al*, 2006). The results of the present study indicate that ulcer healing is compromised in lead exposed rats, as evidenced by a reduced healed ulcer areas on days 14 and 21 when compared with control. Both macro- and macroscopic techniques were used to obtain qualitative and quantitative results. Lead exposure was also reported by Carmouche and others to cause delay in bone fracture healing in mice (Carmouche *et al*., 2005).

It has been shown that proper healing progression requires a decline in gastric acid secretion as healing progresses (Burget *et al*, 1990). Howden and Hunt (1990) investigated the relationship between the suppression of acidity by antisecretory drugs for the treatment of benign gastric ulcer and their corresponding ulcer-healing rates. they observed that for a variety of antisecretory drug regimens, there was a significant correlation between suppression of 24-h intragastric acidity and ulcer healing rates after 2, 4 and 8 weeks of treatment. These findings were buttressed by subsequent reports of McIsaac,*et al* (1991) and Olaleye *et al* (2012). In the present study, the rates of suppression of basal and histamine stimulated gastric acid were lower in the rats exposed to lead when compared with the control. This may partly explain the reduced healing rates in the exposed rats.

Goblet cells, described originally to be mucous producing cells of the intestinal mucosa, are also known to be responsible for production of Intestinal Trefoil Factor (ITF). Several evidences have been put forward to show that ITF is involved in the sequence of events leading to re-epithelialization of the wounded mucosa, by stimulation of epithelial cell motility (Mashimo *et al*, 1996; Milani and Calabro, 2001; Harathi and Jerzy, 2015). The finding in this study of increase in goblet cell counts as healing progresses buttressed previous reports highlighted above. It is known also, that goblet cells secrete mucus that protects the gastric mucosa from the effect of gastric acid and pepsin which are the major causative factors for ulcers. The significantly lower goblet cell counts seen in treated animals may also be a mechanism through which lead

exposure delays gastric ulcer healing in rats as the mucus produced by these cells are very important in protecting the gastric mucosa from the aggressive factors during healing It may however be premature to attribute the changes in goblet cell counts to either ITF or mucous as further studies are needed to identify the exact effect of lead exposure on goblet cell function.

In conclusion, the results of this study have shown that gastric ulcer healing is reduced in lead exposed animals and has further buttressed previous assertions that environmental factors such as exposure to heavy metals and other toxicants may contribute to the global increase in the incidence and relapse in healing of gastrointestinal inflammatory disorders.

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