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Research Article

Age-Related Changes in the Response of the Rat Gastric Mucosa to Acetic acid- and Indomethacin-induced Ulceration

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Abstract

Reduced mucosal protection is known to occur in the gastrointestinal tract of older people. However, the understanding of the repair process in the injured stomach of the aged is low. In this study, the response of the gastric mucosa in rats of different ages to acetic acid and indomethacin induced ulcers were investigated. Male Wistar rats (aged 3, 6, 12 and 18 months) were divided into four groups according to their ages. Two experimental ulcer models- acetic acid (via intraluminal injection of 0.2 mL acetic acid into the glandular portion of the stomach for 45 seconds) and indomethacin (via 25mg/kg body weight of indomethacin, p.o) - were studied in each group. Indices of ulcer healing determined on days 3, 7, 14 and 21 post-induction were: ulcer area by planimetry, ulcer depth and width by histomorphometry, tissue regeneration by histology Gastric acid secretion was measured by continuous perfusion of the stomach with normal saline (1ml/minute) under a mixture of xylazine (0.0005ml/g b.w) and ketamine (0.0015ml/g b.w) anaesthesia (0.6 mg/100 g).. The percentage area of ulcerated stomach healed three days after acetic acid ulcer induction was 37.9, 28.0, 23.6 and 26.9 for the 3, 6, and 12 and 18 month old rats respectively. By day 21 total healing (100%) was observed in the 3month old rats while healing rates were 94.3%, 71.7% and 69.1% for the 6M, 12M and 18M rats respectively. Healing rate also decreased with age in the indomethacin ulcer model. Acid secretion rate declined with age. Significant reduction in acid secretion were observed in the later stage of the healing process and more prominent in younger than older rats. Histology revealed gastric ulceration in all groups on days 3 and 7 with presence of inflammatory cells at ulcer bed. By day 14, reduction in the inflammatory cells and greater fibroblast proliferation were observed in the 3 and 6-month but not in the 12- and 18-month old rats. The study provides experimental evidence for the delayed healing of gastric injury reported in old age.

Keywords: Age, gastric ulcer, healing, histology, histomorphometry, gastric acid

INTRODUCTION

Peptic ulcer disease (gastric and duodenal ulcer) is characterized by an imbalance between gastric offensive factors like gastric acid and pepsin secretion, lipid peroxidation, nitric oxide, *Helicobacter pylori* and defensive mucosal factors like mucin secretion, mucosal cell shedding, glycoprotein proliferation and anti-oxidant enzymes like catalase, superoxide dismutase and glutathione level (Marshall and Warren, 1984; Prabha *et al.*, 2009).

The gastric mucosa elicits cytoprotective and healing responses to ulcerogens which is aimed at restoring the mucosal back to its normal state. Gastric ulcer healing is a spontaneous, complicated array of different mechanisms working in sequence to correct the imbalance between offensive and defensive factors in the stomach (Perini *et al.*, 2003). The process involves alleviation of aggressive luminal factors, filling of mucosal defect with proliferating and migrating epithelial cells and connective components so as to reconstruct the mucosal architecture (Tarnawsaki *et al.*, 2001). The healing of gastric ulcer is influenced by drug therapy as well as by other factors. There are a number of controlled studies (Baume *et al.*, 1972; Buttler and Gersh, 1975; Taylor *et al.*, 1977; Ciclitira *et al.*, 1979; Sutton, 1982) on the effect (positive and negative/adverse) of various drugs on gastric ulcer healing rate; however, there is little documentation on the effects of factors other than drug treatment on the healing of gastric ulcer, such factor is age.

Age has been implicated in the pathogenesis of chronic diseases such as diabetes mellitus and artherosclerosis (Doll, 1995; Albright, 2008). This may be because many hormones associated with the maintenance of immune function decline with advancing age (Pawelec *et al.*, 2002; Arlt and Hewison, 2004). Previous studies have found a reduction of protective physiological factors in gastric mucosa such as prostaglandins, mucus, hormones, including serum gastrin in the elderly population (Khalil *et al.*, 1988; Majumdar *et al.*, 1988). Older individuals often have reduced acid secretion (Maitra *et al.*, 1988; and Ohno *et al.*, 1988), blood flow and prostaglandin levels in the gastric and duodenal mucosa (Masuda *et al.*, 1991; Cryer *et al.*, 1992; Lee and Feldman, 1994; Okabe and Amagase, 2005) as well as reduction in bicarbonate secretion (Kim *et al.*, 1990) and mucosal cell proliferation (Majumdar *et al.*, 1988). However, the role of age and probable pattern of its effect on the spontaneous repair of gastric mucosa against ulcerogens are not clear. In this preliminary report, we examined, using morphometric and histological techniques, the effect of age on the rate of spontaneous healing response of gastric mucosa of male rats of different ages to ulcerogens.

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MATERIALS AND METHODS

Animals

Wistar rats (ages 3, 6, 12 and 18 months) were used in the study. Animals were bred specially to ascertain their ages with the parent stock obtained from the Central Animal House, University of Ibadan, Nigeria. They were kept in wire meshed cages and fed with standard commercial rat pellets (Ladokun Feeds Limited, Nigeria) and allowed water *ad libitum* prior to the start of the experiment. The rats were thereafter separated into four groups according to their ages. All animals received humane care and procedures in this study conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding principles in the care and use of animals, (WMA, APS., 2002).

Induction of Experimental ulceration

Acetic acid-induced ulceration

Gastric ulcers were induced using acetic acid following the method described by Tsukimi and Okabe [1994] with a little modification. After 24 hours of fasting, the rat's stomach was exposed under anesthesia [mixture of xylazine (0.0005ml/g Body weight) and kethamine (0.0015ml/g BW)], a laparatomy was performed through a midline epigastric incision, The glandular walls of the stomach were clamped with a pair of eye forceps rings, and 0.2 ml of acetic acid solution (40% v/v distilled water) was injected into the intra-luminal glandular portion of the stomach, and then withdrawn after 45 seconds. The abdomen was sutured, and rats were allowed to recover. All the animals were fed normally throughout the experiment. Samples were collected on days 3, 7, 14 and 21 post inductions. Gastric lesions were evaluated by examination of the inner gastric surface with a dissecting magnifying glass. The macroscopic ulcer area (mm^2) was determined as described by Takagi *et al*. [1969].

Indomethacin –induced ulceration

Indomethacin was suspended in 1% carboxyl methylcellulose in water and administered by gavage at the dose of 25 mg/kg body weight [Ozbakis-Dengiz *et al.,* 2012]. Samples were collected on days 3, 7 and 14 post inductions. After sacrifice of rats in this study, the abdomen was incised and irrigated with normal saline. Subsequently, the stomach was incised along the greater curvature and then washed gently in running tap water. Ulcers were independently assessed and scored by two observers using the method described by Pihan *et al.*, [1987] according to the following scale: 0 = normal gray coloured stomach, 0.5 = pink to red coloration of stomach, 1 = spot ulcer, 1.5 = hemorrhagic streak, 2 = number of ulcers <5, 3 = number of ulcers >5, 4 = ulcers with bleeding. Ulcer index was calculated by adding the total number of ulcers plus the severity of ulcer.

Determination of lipid peroxidation status

Assessment of lipid peroxidation was carried out following the procedure described by Varshney and Kale (1990). It is based on the reaction of malondialdehyde (MDA) produced during lipid peroxidation with thiobarbituric acid (TBA) forming a pink coloured MDA-TBA adduct that absorbs strongly at 532nm. Animals were sacrificed after the experimental period, and the stomach of each rat was dissected. Stomach from each animal was cut open along the greater curvature, rinsed with normal saline, the mucosa of the ulcerated area was scraped and homogenised in phosphate buffer (tissue to buffer ratio - 1:3), 0.2ml of test sample was added to 0.8ml of Tris-KCl. The solution was quenched with 0.25ml of TCA. 0.25ml of TBA was then added and the solution was then incubated for 45minutes at 80%C. A pink coloured reaction mixture was formed. The reaction mixture was then centrifuged at 1400 rpm for 15 minutes. The absorbance of the supernatant was read at 532 nm. MDA was calculated for each sample as described in a previous report by Adeniyi *et al* (2014).

Histomorphometry

On days 3, 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]. Haematoxylin and eosin stained stomach tissue sections were subjected to morphometric analysis recommended by World Health Organization WHO [1991] which included: dividing the eye piece occulometer 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 (occulometer) was 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). Calibration was needed for each microscope use. The occulometer fixed into the Olympus® microscope was then focused through stained sections of the tissue to allow for the measurement of the depth of ulcer and thicknesses of muscularis external.

Histological processing and examination

Histological study was carried out as described by Ogihara and Okabe [1993]. Small section of stomach were taken from two distinct areas from each stomach and placed in 10% formalin for histological examination. The stomach was fixed, cut into 5 µm sections, stained with hematoxylin 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.

Gastric acid secretion studies

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. Animals were anesthetized with a mixture of xylazine (0.0005ml/g b.w) and ketamine (0.0015ml/g b.w). The animal was then placed on the dissecting board and the limbs tied to the board. The trachea was located and isolated around the neck; it was cannulated and slightly opened to aid breathing. The stomach was then located and a cannula placed in it from the duodenal end of the stomach as previously described. [30] Normal saline (9g of sodium chloride in 1litre of distilled water) was then passed to the stomach from the mouth through the aid of a

cannula. The gastric content of the stomach was collected after every 10 minutes. This was for the basal recording of gastric acid. After about three different recordings, histamine was injected into the animal's system via the hepatic portal vein. The gastric content was also collected after every 10 minutes and three readings were collected for the stimulated acid secretion.

The acidity of each 10 minutes effluent collected was assayed by titration. Sodium hydroxide (1ml of NaOH was dissolved in 399mls of distilled water) and titrated against 10mls of gastric collection after every 10 minutes. The change in volume on the burette was then recorded for each titration, while the indicator used for the titration was phenolphthalein and the pH of gastric effluent was also determined using pH meter. After the treatment period, rats were sacrificed through cervical dislocation and dissected, blood samples were taken via cardiac puncture and the stomachs were collected for determination of lipid peroxidation status and histological studies.

Statistical analysis

All values are presented as Mean ± SEM (standard error of the mean). The statistical significance of differences among groups was assessed using one-way ANOVA and value of $p<0.05$ was considered significant.

RESULTS

Effect of Age on ulcer healing rates

Administration of acetic acid and indomethacin induced gastric ulcer. In particular, the acetic acid model results in ulcers of consistent size and severity at incidence of 100%, and highly resemble human ulcers in terms of both pathological features and healing mechanisms.

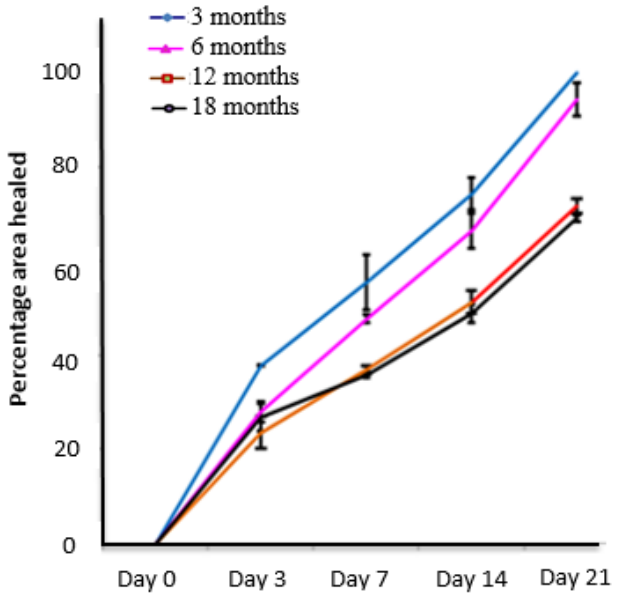

Fig. 1:
Percentage area of gastric tissue healed after acetic acid induced ulcer

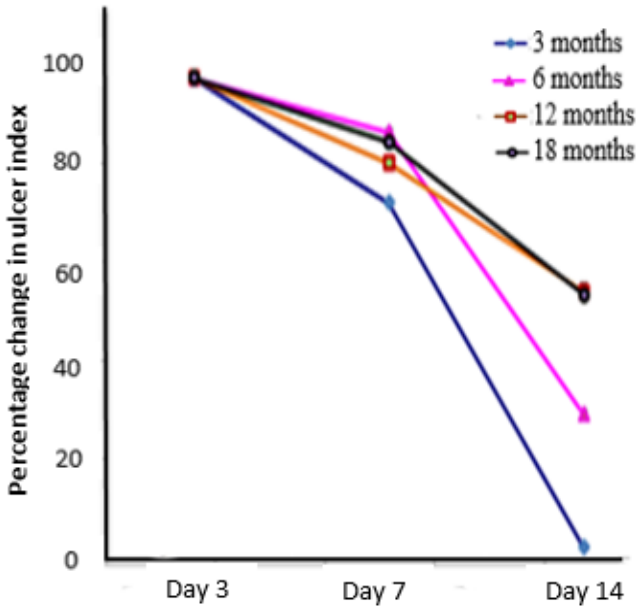

Fig. 2:
Percentage change in ulcer index of rats following induction of gastric ulcer with indomethacin in reference to day 3 after ulcer induction

Figure 1 shows the effect of age on the rate of ulcer healing. Three days after ulcer induction, the proportions of the acetic acid-ulcerated area healed were 37.9, 28.0, 23.6 and 26.9 for the 3, 6, and 12 and 18 month old rats respectively. The 3 months old rats with the same ulcerogen had complete healing 21 days post-induction, while healing rates were 94.3%, 71.7% and 69.1% for the 6, 12 and 18 months rats respectively.

Similar pattern of reduced rate of healing with increasing age of rats was observed in the indomethacin model of ulcer induction (Fig. 2). Specifically, the 3, 6, 12 and 18 months old rats had 2.5%, 30.2%, 55.7% and 55.0% ulcerated unhealed area 14 days post induction.

Effect of Age on ulcer depth during healing:

The effects of age on ulcer depth during the healing of gastric ulcer induced by acetic acid and indomethacin are shown in Fig. 2. Ulcer depth three days after acetic acid induced ulcer (for all age groups) was 1.50±0.000µm. On day 21 post acetic acid induced ulcer, ulcer depth had reduced to 0.26±0.060 µm in the 3 months old rats, 0.44±0.060 µm in the 6 months old rats. The 18- and 21-month old rats had ulcer depths of 1.00±0.000 µm and 1.10±0.100µm respectively (Fig. 2a).

On day 14 after induction of ulcer with indomethacin, the ulcer depth reduced to 0.10±0.000 µm in 3 months old rats, 0.20±0.020 µm in 6 months old rats, 1.00±0.000 µm in 12 months old rats and 1.00±0.000 µm in 18 months old rats (Fig. 2b).

In general, the results showed that younger rats have a lower ulcer depth values compared to the older rats value as healing progress.

Effect of Age on gastric mucosal thickness during healing:

As shown in Fig. 3, gastric mucosa thickness was higher in younger rats compared to the older rats as healing progress for both ulcer models.

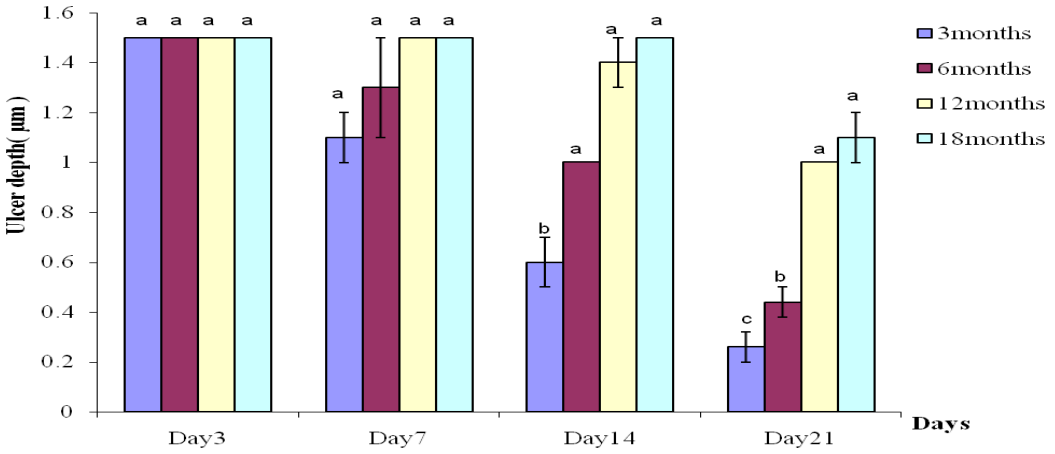

Figure 3
Ulcer depth in rats following induction of gastric ulcers with acetic acid.
Each bar represents Mean +SEM of 5 animals. Bars carrying different letters are statistically different at p<0.05

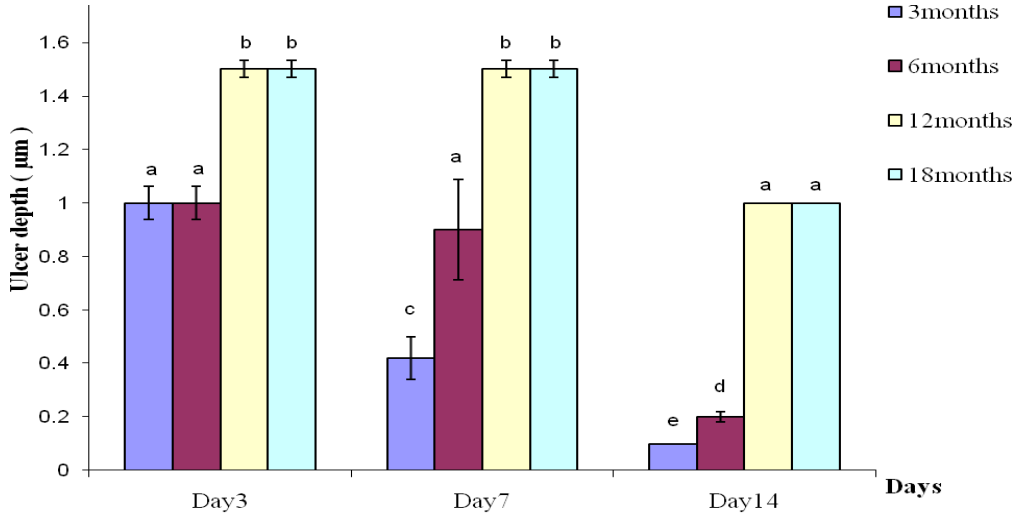

Figure 4
Ulcer depth in rats following induction of gastric ulcers with indomethacin.
Each bar represents Mean +SEM of 5 animals. Bars carrying different letters are statistically different at p<0.05

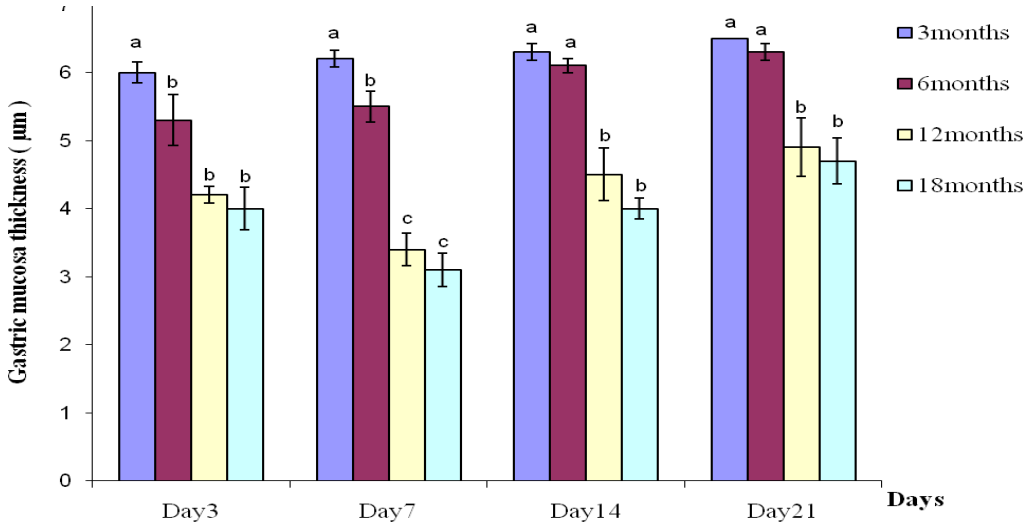

Figure 5
Gastric mucosal thickness in rats following induction of gastric ulcers with acetic acid
Each bar represents Mean +SEM of 5 animals. Bars carrying different letters are statistically different at p<0.05

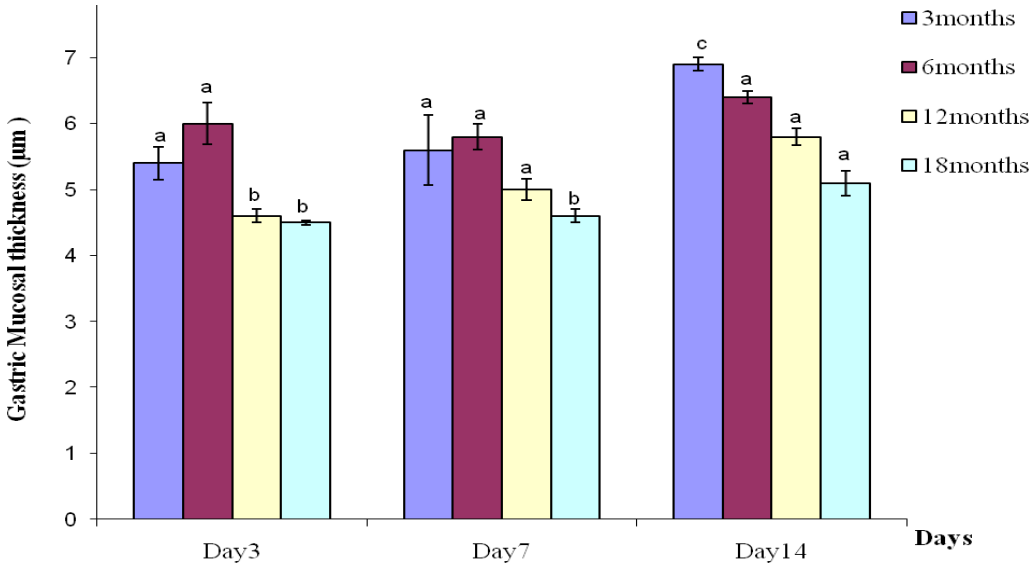

Figure 6
Gastric mucosal thickness in rats following induction of gastric ulcers with indomethacin
Each bar represents Mean +SEM of 5 animals. Bars carrying different letters are statistically different at p<0.05

Table 1:
Basal gastric output in rats following induction of gastric ulcer with Acetic Acid (Mean ± SEM in mEq/L X 1000)

Groups	Control (Pre-ulcer)	Day 3	Day 7	Day 14	Day 21
3 months	0.71±0.01	1.17±0.006[a]	1.21±0.023[a]	1.28±0.014[e]	1.19±0.018[a]
6 months	0.70±0.08	0.95±0.016[b]	1.19±0.005[a]	1.25±0.019[e]	1.16±0.016[a]
12 months	0.65±0.01	1.01±0.037[c]	1.21±0.016[a]	1.21±0.004[a]	1.15±0.016[a]
18 months	0.50±0.05	0.91±0.019[d]	1.14±0.035[a]	1.18±0.004[a]	1.11±0.001[f]

Superscripts a,b,c,d,e,f = different letters are showing that figures are statistically different at p<0.05)

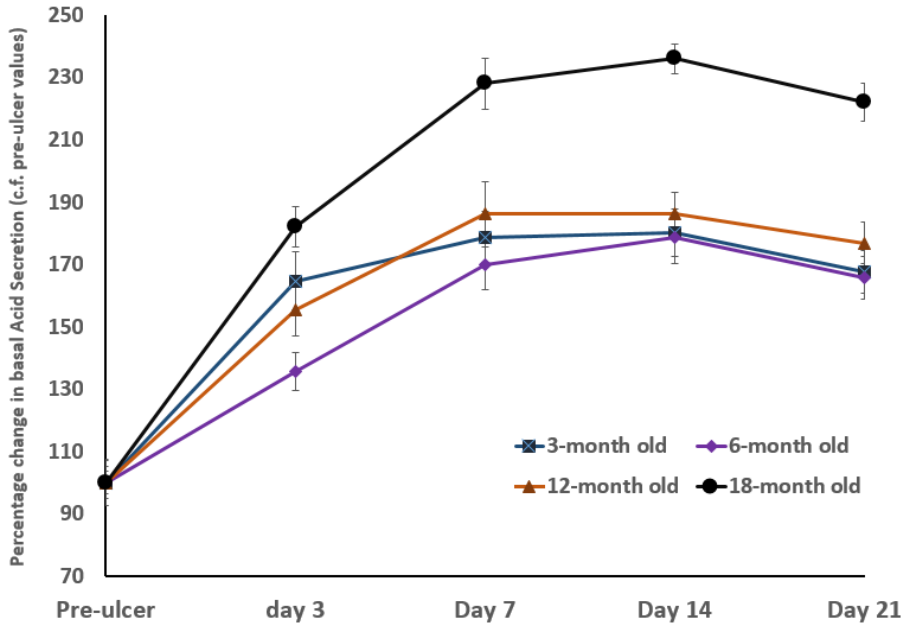

Fig. 7
Percentage change in gastric acid secretory output (over the pre-ulcer values) 3, 7, 14 and 21 days after induction ulcer by acetic acid.
Each point represent Mean± SEM of 5 animals.

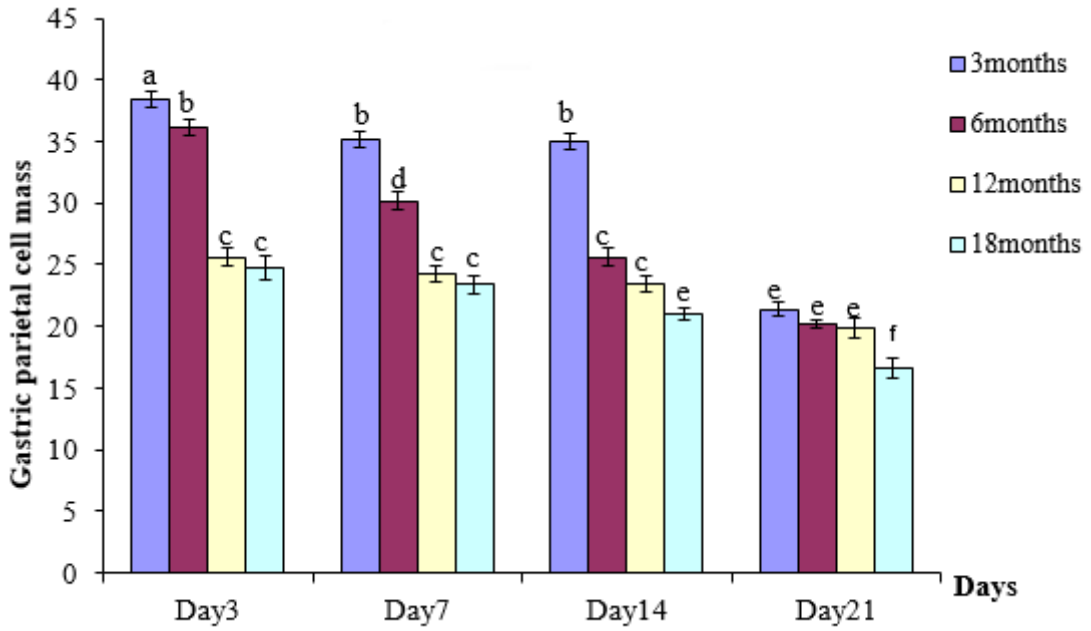

Figure 8:
Gastric parietal cell mass of rats following induction of gastric ulcer with acetic acid.
Bars carrying different letters are statistically different at p<0.05

Effect of age on gastric acid secretion in normal and ulcerated stomach

As shown in Table 1, the rate of secretion of gastric acid decreased with the age of the animals (from 0.71±0.01mEq/L/min in the 3-month old rats to 0.50+0.05 mEq/L/min in the 18-month old rats). The Table also shows that induction of ulcer by acetic acid resulted in significant increases in acid secretory rates in all the animals on days 3, 7 and 14 days post-ulcer, but started declining towards pre-ulcer rate by day 21. The rate of change in acid secretion over the pre-induction stage is shown in Figure 7

Effect of age on parietal cell mass in normal and ulcerated stomach

Figure 8 shows the changes observed in the parietal cells during the healing of gastric ulcer induced by acetic acid. Generally, parietal cell counts decreased in all age groups, and the number of parietal cell decreased with advancing age during the healing period.

Histological evaluation of the effect of age on gastric ulcer healing rate.

Acetic acid model: Plate 1 shows the results of the histological examination on the effect of age on gastric ulcer healing rate following induction of ulcer with acetic acid, showing the mucosa, sub mucosa and muscularis layer of the stomach wall. Histograph of stomach of control rats showed a normal architecture of the mucosa surface for all the age categories. But on day 3, histograph of 3 months old rats stomach showed a wide sub-mucosal oedema and ulceration, while 6 months old rats stomach showed extensive sub mucosal oedema and ulceration, complete mucosal necrosis and sub mucosal oedema was observed in the 12 months old rats, and an area of intense inflammation seen in the 18 months old rats.

The histograph of the three months old rat stomach seven days after ulcer induction showed surface and part of deeper mucosal ulceration, while extensive ulceration with onset of necrosis was seen in those of the six month old rats. While extensive fibrosis with inflammation was seen in the 12 months old rats, the photomicrographs from the 18 months old rats revealed necrotic mucosa and deeper ulceration. Fourteen days post-ulcer, mild vascularisation with sub mucosal oedema was seen in gastric tissues of the 3 months old rats, while focal areas of ulceration were observed in 6 months old rats. Mild mucosal ulceration with inflammation in 12 months old rats, but sub mucosal oedema, and inflammation in the 18 months old rats.

Indomethacin model: Plate 2 shows the results of the histological examination on the effect of age on gastric ulcer healing rate following induction of ulcer with indomethacin, showing the mucosa, sub mucosa and muscularis layer of the stomach wall. While the sub mucosa and muscularis mucosal layers from the stomach of control (ulcerated) rats showed normal architecture, observable changes were seen in stomach of the ulcerated rats. Three days after indomethacin challenge, stomach samples from the three months old rats showed ulcerated mucosa with mild Sub mucosal oedema; mucosal hyperplasia and ulceration were observed in the 12 months old rats while areas of deep ulceration were seen in the 18-month old rats. The histologic observations seven days after indomethacin induced ulcer include focal mucosal ulceration in 3 months old rats, focal areas of deep and surface mucosal ulceration in 6 months old rats, focal area of deep mucosal ulceration in 12 months old rats and area of mild surface ulceration in 18-months old rats stomach histograph.

While complete healing was evident in the 3-month old rat stomach after 14 days of indomethacin challenge (as evidenced by normal submucosa) the histograph of stomach of predominantly mild surface mucosa ulceration were seen in slides from the six-month old rats. Deep and mild surface mucosa ulceration were evident in 12-month and 18-month old rats.

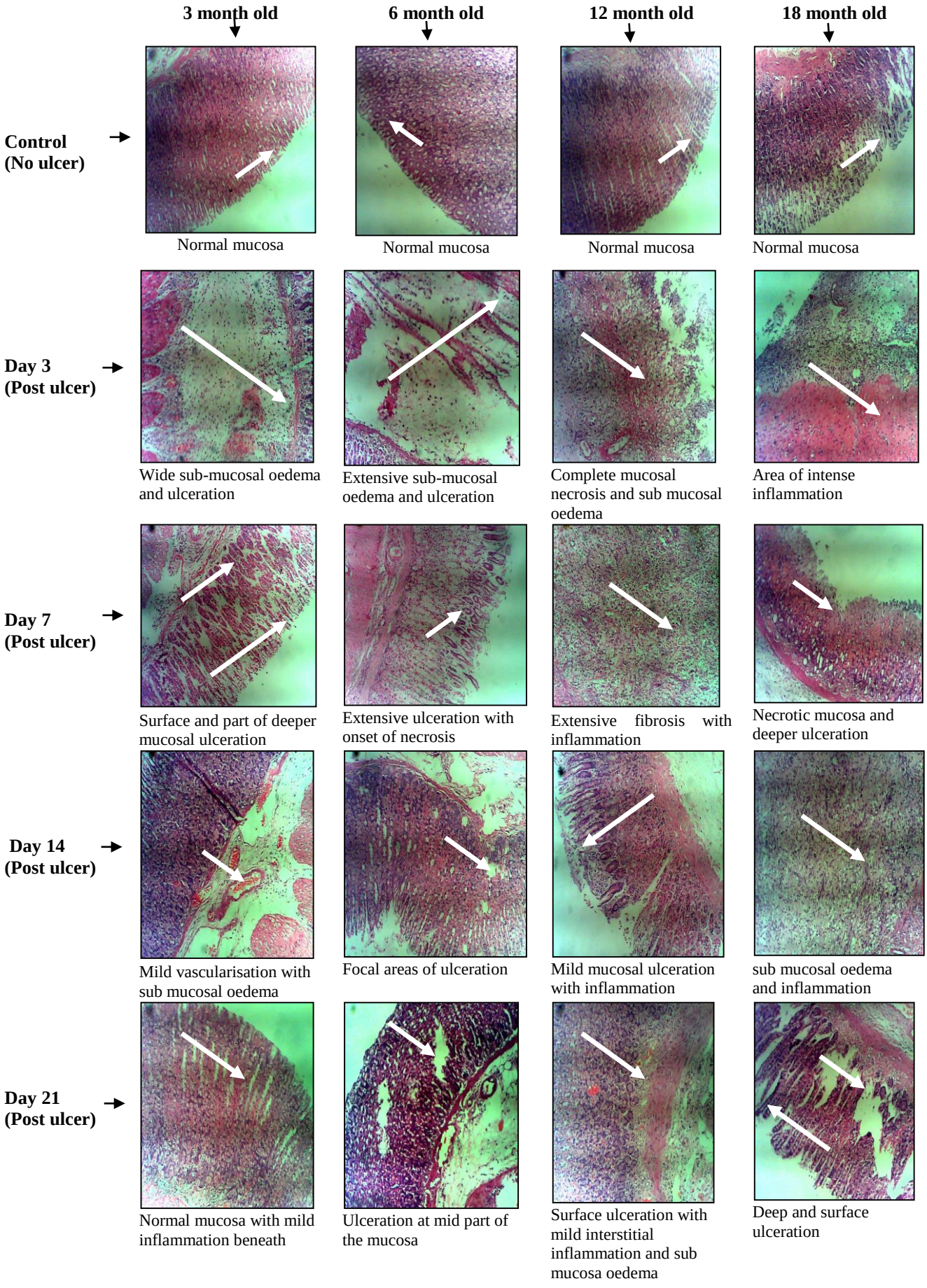

Plate 1
Photomicrograph collage of the stomach of 3-, 6-, 12- and 18-month old rats before and after acetic acid induced ulceration. Repair processes towards normal stomach were observed in the 3- and 6-month old but not in the 18-month old rats. H & E, X10.

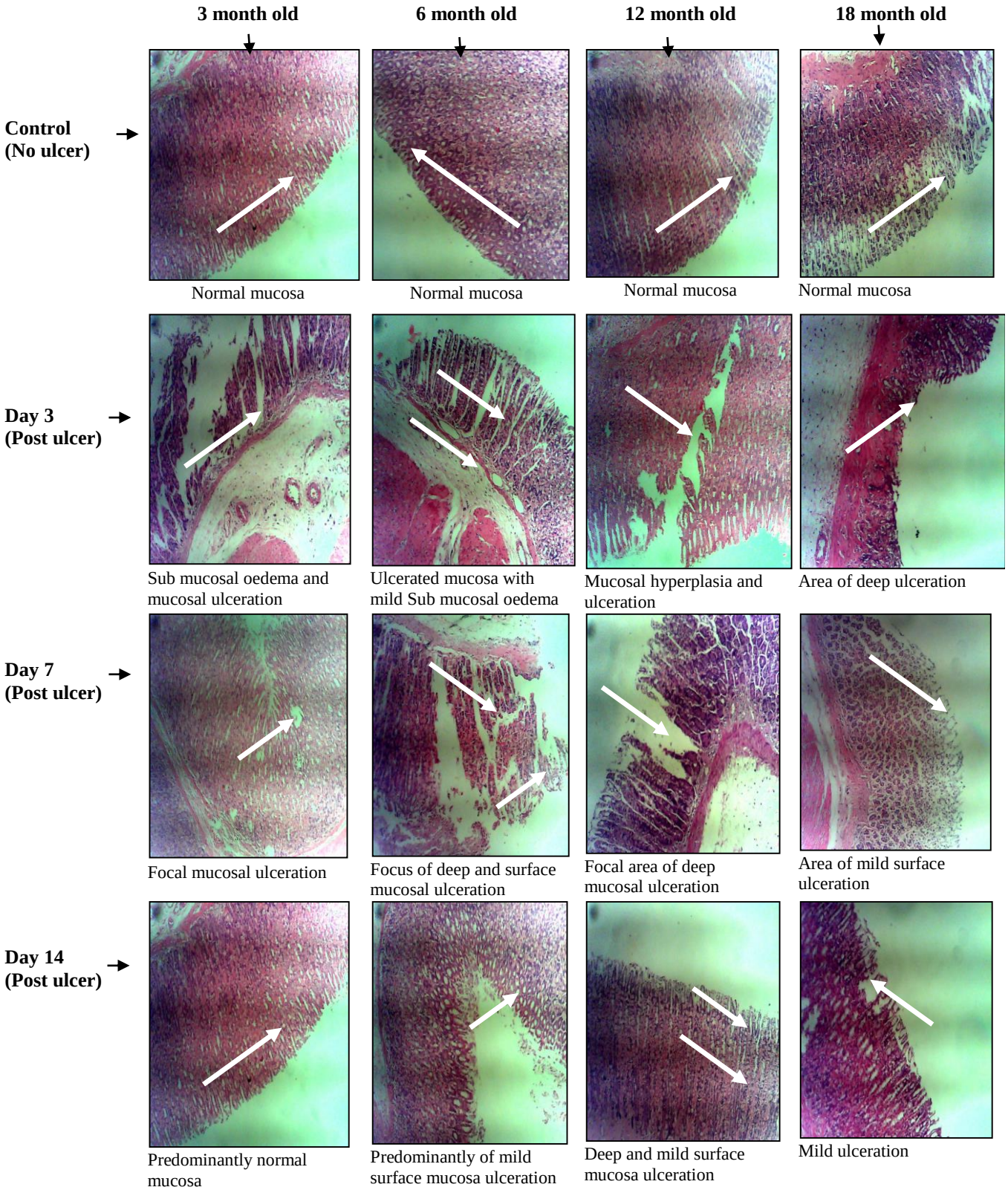

Plate 2
Photomicrograph collage of the stomach of 3-, 6-, 12- and 18-month old rats before and after indomethacin induced ulceration with similar patterns of repair observed with acetic acid ulcer model. H & E, X10.

DISCUSSION

Available demographic data tend to suggest a steady growth in the population of the aged growing globally (Christensen et al, 2009). Therefore, understanding of the effect of ageing on physiology and pathophysiology of diseases will be of tremendous importance in global health and disease control. It has long been observed clinically that gastric ulcers occur more frequently among persons more than 60 years of age and that the size of gastric ulcer in older patients is appreciably

larger than in younger individuals in response to histamine-2 receptor antagonist, famotidine (Blanco and Domenico, 1993). These observations have been attributed to deficiencies in the healing process of old individual compared to the younger ones. In this study, we used both acetic-acid induced gastric ulcer a chronic ulcer model, because it accurately reflects human peptic ulcer disease (Okabe and Amagase, 2005) and indomethacin induced gastric ulcer an acute ulcer model because of its direct local toxic effects on the gastrointestinal mucosa (Whittle, 1998). In this study, administration of acetic acid and indomethacin to the gastric mucosa of male rats of different ages resulted into formation of ulcerated stomach areas.

Gastric ulcer is characterized by the imbalance between gastric offensive factors (like pepsin secretion, lipid peroxidation, nitric oxide) and defensive mucosal factors (like glycoprotein proliferation, and antioxidants like catalase, superoxide dismutase and glutathione level) (Prabha *et al.,* 2009). The healing of this gastric ulcer involves a complex process of restoring the mucosal to its normal state by correcting the imbalance between the aggressive and defensive factors in the stomach (Perini *et al.,* 2003).

Many studies involving the gastrointestinal tract are made in rodents without giving adequate consideration to the age of the animals. Interpretations and extrapolations in such findings become difficult because metabolic processes, drug interactions and a number of body activities are known to be influenced by age. In a report correlating age of rats with those of humans, Sengupta (2013) agreed with figures from previous studies which indicate that in the pre-pubertal stage, 4.2 rat days is equivalent to one human year while in the aged stage, 17.1 rat days equals one human year (Quinn, 2005; Sengupta, 2012). The implication of these rat/human age correlations is that the equivalent human ages used in the present study ranged between 9 and 60years. The results of this study showed that healing of ulcerated gastric mucosa was markedly accelerated in younger rats (3 and 6 months old) and delayed in older rats (12 and 18 months old) as revealed by significantly lower ulcerated area, lower ulcer index with ulcer depth as well as higher mucosa thickness seen in younger rats. This could be due to the reduced gastro-protective potential of elderly population gastric mucosa as suggested by previous studies which found a reduction in protective physiological factors in gastric mucosa such as prostaglandins, mucus and hormones, including serum gastrin in the elderly population (Khalil *et al.,* 1988; Majumdar *et al.,* 1988), it could also be that older individuals often have reduced bicarbonate secretion (Kim *et al.,*1990) and mucosal cell proliferation (Okabe and Amagase, 2005; Majumdar *et al.,* 1988) among other conditions. Similarly, the maintenance of the cyto-architecture of gastric mucosa with less intensive mucosal oedema, inflammation and ulceration seen in the younger rats during healing of ulcer suggests that younger rats' stomach have inherent higher anti-inflammatory activities, a property of agents that will have lower incidence of ulceration complications by selectively inhibiting COX-2 enzyme activity (Bombardier *et al.,* 2000; Silverstein *et al.,* 2000).

It has been documented that gastritis and gastric ulcers are associated with stress (Kumar *et al.,* 2010) possibly by inducing lipid peroxidation. This study revealed that the stomach tissue of younger rats prevents lipid peroxidation better, which results into lower Malondialdehyde (lipid peroxidation index). This result implicates oxidative stress in the differences in the rate of healing of gastric ulcer in young and old rats. Further studies on the role of antioxidants and other indices of oxidative stress will however be required before conclusions of the oxidative stress mechanism could be made.

In conclusion, this study provides information on the role of age during spontaneous healing response of the stomach to ulcerogens, showing that the healing rate of gastric ulcer is faster in younger animals when compared with the older ones. The clinical implication of this finding is that the age of patients should be considered an important factor when prescribing drugs that may interfere with the integrity of the gastrointestinal mucosa, as natural repair processes are more likely to be completed in relatively younger individuals when compared with older patients. It is also suggested that conclusions on laboratory studies involving inflammation and erosion of the gastrointestinal tract should not be drawn until age of the animals is factored into the design and results of such experiments.

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