

AGE-RELATED CHANGES IN PROSTAGLANDIN E₂, NITRIC OXIDE, AND VASCULAR ENDOTHELIAL GROWTH FACTOR LEVELS IN GASTRIC MUCOSA DURING THE HEALING OF ACETIC ACID-INDUCED ULCER

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ABSTRACT

Objective: Nitric oxide (NO), prostaglandin E₂ (PGE₂), and vascular endothelial growth factor (VEGF) are fundamental regulators of epithelial restitution and angiogenesis. They play important roles in ulcer healing. Insights into their possible changes during gastric ulcer healing putting age into consideration could give a guide to the proper management of ulcers in the aging population. This study, therefore, examined alterations in the concentrations of PGE₂, NO, and VEGF in the gastric mucosa of rats of different ages after induction of ulcer and during healing.

Methods: Male Wister rats (aged 3, 6, and 18 months old) were divided into three groups according to their ages. The ulcer was induced using the acetic acid ulcer model. Healing indices studied on days 3, 7, and 14 were the macroscopic dimension of ulcer, stomach tissue concentration of PGE₂, NO, and VEGF, with the immunohistochemical expression of VEGF.

Results: Outcome of this study showed 100%, 88.36%, and 62.30% area of mucosa healed in 3-, 6-, and 18-month-old rats respectively, on day 14 post-induction of ulcer. PGE₂, NO, and VEGF concentrations were inversely proportional to age during healing. Immunohistochemical staining showed that younger rat (3 and 6 months old) had higher expression of VEGF throughout the healing period.

Conclusion: It was therefore concluded that the slower rate of healing in older rats could be due to reduced gastroprotection, epithelial restitution, and angiogenesis as age increases.

Keywords: Gastric ulcer healing, Prostaglandin E₂, Nitric oxide, Vascular endothelial growth factor.

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INTRODUCTION

The incidence of gastrointestinal dysfunctions, including gastric and duodenal ulcers, increases with age [1]. This may occur as a result of reduced gastroprotective factors and mechanisms [2], increased susceptibility of the mucosa to various damaging agents such as aspirin use for cardiovascular and cerebrovascular events, prophylactic use of nonsteroidal anti-inflammatory drugs for arthritis and musculoskeletal ailments [3,4], and a consequent impairment to healing processes [5].

The normal gastric mucosa integrity is maintained by defensive factors which include mucus-bicarbonate-phospholipids complex, epithelial cell layer regulated by nitric oxide (NO), prostaglandin E₂ (PGE₂), vascular endothelial growth factor (VEGF), trefoil proteins, heat shock proteins, and continuous mucosal blood flow [6,7].

Pg can stimulate almost all the mucosal defense mechanisms, particularly; they reduce acid output, stimulate mucus, bicarbonate, and phospholipids production, increase mucosal blood flow, and accelerate epithelial restitution and mucosal healing [8]. Pg are also known to inhibit mast cell activation as well as leukocyte and platelet adhesion to the vascular endothelium [8].

NO has been shown to protect the gastrointestinal mucosa from a variety of insults, including injuries from ethanol, mineral acids, bile acids, ischemia-reperfusion, and early endotoxin-induced damage [9], and this is done by maintaining a balanced microcirculation in the mucosa and by inhibiting neutrophil accumulation [10,11], thereby aiding gastric protection and accelerating ulcer healing [12].

Aging gastric mucosa may have an increased susceptibility to gastric ulcer, which may result from overwhelming exposures to aggressive substances in the presence of weaker defensive mechanisms [6,7,13]. Insights to changes occurring during gastric ulcer healing with advancing age could give a guide to the right management of gastric ulcers in the aging population.

Healing of ulcer involves processes such as cell proliferation, migration, re-epithelialization of the gastric gland, development of new blood vessels, and deposition of the matrix which finally result in the formation of scar tissue [14,15]. Gastric ulcer healing can be promoted by expression of VEGF, a growth factor that promotes the formation of granulation tissue and new microvessels through angiogenesis [16-18].

These biomolecules (NO, PGE₂, and VEGF) are fundamental regulators of mechanisms that play important roles in gastroprotection and also in the healing process of ulcer [6,7]. However, age could affect their concentration and function, an area where there is a dearth of information particularly in the process of healing of the gastric ulcer. Therefore, a study to investigate changes in their concentrations and expressions may provide insight into the mechanisms by which age affects gastric ulcer healing. Therefore, this study is aimed at investigating the age-related changes in PGE₂, NO, and VEGF during the healing of acetic acid-induced gastric ulceration.

MATERIALS AND METHODS

Experimental animals

Male Wister rats, aged 3, 6 and 18 months, were obtained from the Institute for Advanced Medical Research and Training, University

College Hospital, Ibadan, Oyo State. The rats were acclimatized for 2 weeks in a well-ventilated room and under a room temperature of 37°C, given rats' pellets and drinking water *ad libitum*.

Ethical statement

All animals received humane care, and the study procedures were in accordance with the laid down principles required for research carried out on animals as suggested by the Helsinki Declaration and the approved guidelines in the handling of animals for experiments [19]. Ethical approval was obtained from the Oyo State Research Ethics Review Committee, the Ministry Of Health Secretariat, Ibadan, Nigeria (Reference number: AD 13/479/459).

Experimental design

Animals were grouped into 3, according to their ages (3, 6, and 18 months old) with 15 rats in each group. Stomach and blood samples were collected on days 3, 7, and 14 after the induction of ulcer with five rats picked from each group on each day. All the animals were fed normally throughout the experiment except before ulcer induction when they are fasted for 24 h.

Induction of gastric ulcer with acetic acid

Gastric ulcers were induced using acetic acid by the method previously described by Ajayi and Olaleye [20]. Briefly, after rats have fasted for 24 h, stomach was exposed by laparotomy under anesthesia which was a mixture of 5% ketamine (35.0 mg/kg b.w.) and 2% xylazine (5.0 mg/kg b.w.) injected intramuscularly, thereafter, the stomach glandular wall was clamped with an eye forceps and 0.2 ml of 40% acetic acid was injected with 1 ml syringe into the gastric intraluminal glandular portion within the portion clamped with the eye forceps, then the acetic acid was drawn back to the syringe after 45 s, and the stomach was bath with normal saline. The abdominal muscles were sutured after the stomach was returned to the abdomen and the skin was sutured too for each animal. The rats were taken back to their cages under close monitoring to recover from the surgical procedure.

Ulcer area estimation

Ulcer areas in mm² were determined on days 3, 7, and 14 post-induction of gastric ulcer with acetic acid. The rats were euthanized on each of these days, after which the stomach of each rat was separated, cut opened along the greater curvature, rinsed with normal saline, then spread out, and pinned on a cork board. The ulcer area was measured using a ×2 magnification hand lens and calculated using the collection of guiding principles of drug administration of The Ministry of Health Beijing, 1993, [21] with the formula below:

$$\text{Ulcerated area (mm}^2\text{)} = \pi (d1/2) \times (d2/2),$$

Where d1 represents the longest longitudinal diameter of the ulcer and d2 represents the longest transverse diameter of the ulcer.

The percentage area of ulcer healed was determined as described by Adeniyi *et al.* [22]

Percentage area healed on the day 7=

$$\frac{\text{Area of an ulcer on day 3} - \text{the area of an ulcer on day 7}}{\text{Area of an ulcer on day 3}} \times 100$$

Percentage area healed on the day 14=

$$\frac{\text{Area of an ulcer on day 3} - \text{the area of an ulcer on day 14}}{\text{Area of an ulcer on day 3}} \times 100$$

Preparation of stomach homogenate and Estimations of NO, PgE₂, and VEGF

Tissue samples from the glandular portion of the stomach were excised, washed, and homogenized in ice-cold 0.1M phosphate buffer

(1:4 (w/v), pH7.4), and homogenates were centrifuged at 2500 rpm for 10 min at 4°C. Determination of NO, PgE₂, and VEGF was done using the supernatants obtained.

Determination of NO concentration was done by the determination of nitrate and nitrite, and the concentration of nitrate was determined with Griess reaction as described by Tsikas [23]. Nitrate was measured through hydrazine sulfate reduction using the method of Chen and Hu [24]. PgE₂ and VEGF concentrations were measured using the ELISA method by following the kit manufacturer's guide (Elabscience, China).

Histopathological studies

Tissues from the glandular portions of the excised stomachs of animals from each group were washed in phosphate-buffered saline and kept in 10% formaldehyde solution for histological studies. Prepared sections of tissue were inserted in paraffin and stained after deparaffination using hematoxylin and eosin stain (H and E) to verify morphological formations and immunohistochemical assessment [25].

Immunohistochemistry of VEGF

Determination of the expressions of VEGF in gastric mucosa cells was carried out by immunohistochemistry procedure using anti-rat VEGF antibody (BIOCOM Biotech, South Africa) exposed to streptavidin-peroxidase as previously described by Gillett *et al.* [26].

Labeling index calculation from immunoratio web application

ImmunoRatio web application (<http://jvsmicroscope.uta.fi/immunoratio/>) for Image J (<http://imagej.nih.gov/ij/>) which resides in a remote server accessed through the internet with a web browser was used to quantify the percentages of positively stained nuclei for VEGF. The main properties of the web application include separating diaminobenzidine-stained (DAB) area from hematoxylin-stained regions of the image, calculating the percentage of a DAB-stained region over the total region, known as the labeling index, and it also generates a pseudo-colored image corresponding with the area segmentation [27].

Statistical analysis

Analysis of data was done using a statistical software GraphPad prism (version 5.0). Expression of results was done as a mean ± standard error of the mean, while statistical significance was assessed with one-way analysis of variance. The differences at p<0.05 were considered to be statistically significant.

RESULTS

Gastric ulcer area post-induction with acetic acid

Table 1 shows the gastric ulcer area in rats during the healing of gastric ulcer induced with acetic acid. The 3-month-old rats had the lowest gastric ulcer area compared to both the 6-month-old and 18-month-old rats during healing.

The percentage area of ulcer healed on days 7 and 14 post-induction

Fig. 1 shows the percentage area healed in rats after gastric ulcer induction with acetic acid on days 7 and 14 of ulcer healing. The 3-month-old rats had the highest percentage area healed compared to 6- and 18-month-old rats.

Concentration of PgE₂ in gastric tissue post-induction of ulcer

Fig. 2 shows the PgE₂ concentration in the stomach tissue of rats during the healing of gastric ulcer induced with acetic acid. PgE₂ increased

Table 1: Gastric ulcer area post-induction with acetic acid

Age of Rats	Day 3	Day 7	Day 14
3 months	6.62±0.18 ^a	3.36±0.10 ^d	0.00±0.00 ^f
6 months	7.56±0.08 ^b	5.42±0.07 ^e	0.88±0.10 ^g
18 months	9.24±0.11 ^c	7.44±0.16 ^b	3.48±0.07 ^d

Superscripts a, b, c, d, e, f, g: Different letters are showing that figures are statistically different at P<0.05

during healing; also PgE₂ concentration was higher in the 3-month old and 6-month old rats compared to 18-month old rats particularly on days 7 and 14.

NO concentration in stomach tissue post-induction of ulcer

Fig. 3 shows the NO concentration in the stomach tissue of rats after the induction of gastric ulcer with acetic acid. NO concentration was not significantly ($p>0.05$) different across the age groups on days 3 and 7, but on day 14, NO concentration was inversely proportional to age and the differences were statistically significant ($p<0.05$).

VEGF concentration in stomach tissue post-induction of ulcer

Fig. 4 shows the VEGF concentration in stomach tissue in rats during the healing of the acetic acid-induced gastric ulcer. The stomach tissue homogenate concentration of VEGF in 3- and 6-month-old rats was higher compared to that of 18-month-old rats during healing.

Gross pictures of the stomach post-gastric ulcer induction with acetic acid

Fig. 5 shows the gross pictures of the stomach tissue in rats during the healing of gastric ulcer induced with acetic acid after 3, 7, and 14 days' post-ulcer induction for 3-, 6-, and 18-month-old rats. On day 3, the 3-month-old rats showed lesser hemorrhagic streaks than the 6- and 18-month-old rats. While on day 14 the 3-month old rats had a normal mucosa with no evidence of ulceration, the 6-month old rats also showed normal mucosa but with lesser mucus covering than in 3-month-old rats, while the gastric mucosa of 18-month-old rats showed evidence of spot ulcers.

Photomicrograph collage of the stomach histology post-induction of gastric ulcer with acetic acid

Fig. 6 shows the photomicrograph collage of the stomach histology of rats on days 3, 7, and 14 post-gastric ulcer inductions with acetic acid.

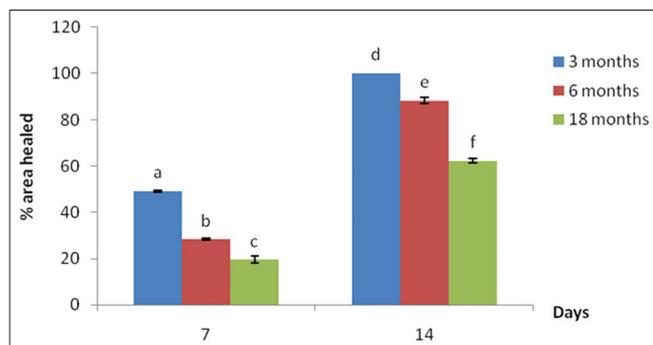


Fig. 1: Percentage area of ulcer healed on days 7 and 14 post-induction. Bars carrying different letters are statistically different at $p<0.05$

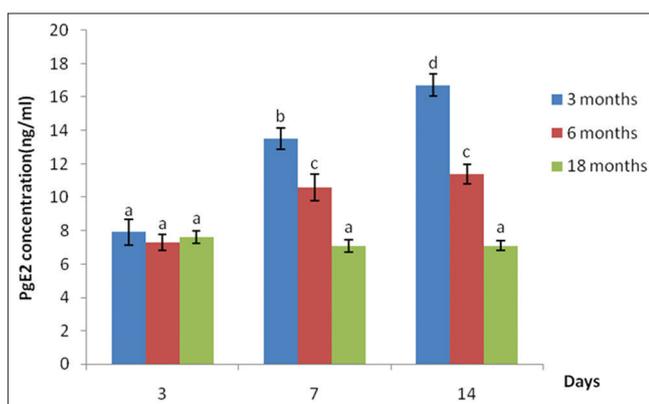


Fig. 2: Concentration of prostaglandin E2 in gastric tissue post-induction of ulcer. Bars carrying different letters are statistically different at $p<0.05$

Photomicrograph of VEGF expression

Figs. 7 and 8 show the photomicrograph and percentage intensity of VEGF expressions in rats during the healing of acetic acid gastric ulcer model. After 3 days of gastric ulcer induction, the expression of VEGF was 66.4%, 56.3%, and 48.8% for the 3-, 6-, and 18-month-old rats, respectively. After 7 days of gastric ulcer induction, the expression of VEGF was 68.2%, 67.2%, and 44.6% for 3-, 6-, and 18-month-old rats, while on day 14 after induction of ulcer, the expression was 79.9%, 74.8%, and 54.5%. This result showed that the 3-month-old rats had the highest expression of VEGF when compared with the 6- and 18-month-old rats post-induction of gastric ulcer.

DISCUSSION

Previous studies have reported that the aging gastric mucosa is more susceptible to ulcer formation [28,20] due to an impaired mucosal defense [4,29]. During ulcer healing, factors such as VEGF, PgE₂, and NO are known to play fundamental roles in the regulation of angiogenesis, an important process required for healing to take place [30].

This present study investigated the age-related differences in VEGF, PgE₂, and NO concentrations during the healing of the acetic acid-induced gastric ulcer. This gastric ulcer model produces lesions that resemble the human gastric ulcer in its severity and duration, therefore it is considered suitable for gastric ulcer healing studies [28,31].

This study showed an accelerated healing rate of gastric ulcers in 3- and 6-month-old rats and delayed healing rate in 18-month old rats, implying that healing was inversely proportional to age, and this is evidenced by the results of ulcer areas and the percentage ulcer area healed and may be due to a reduction in gastroprotective factors as an organism increases in age [2]. This result was similar to result obtained

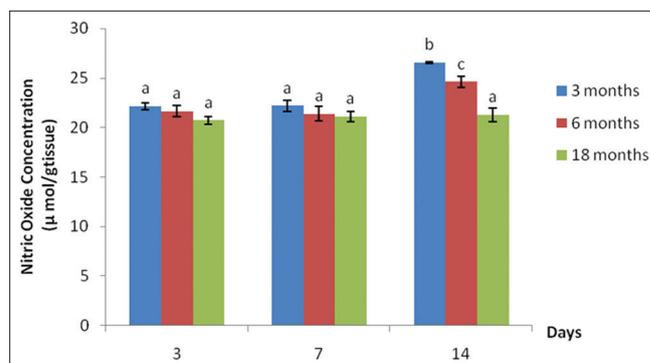


Fig. 3: Nitric Oxide concentration in stomach tissue post-induction of ulcer. Bars carrying different letters are statistically different at $p<0.05$

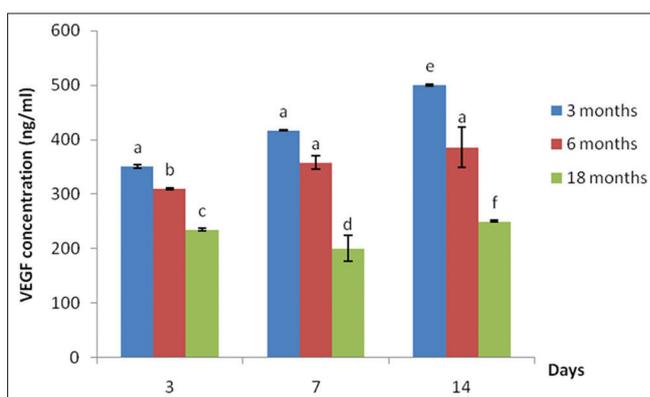


Fig. 4: Vascular endothelial growth factor concentration in stomach tissue post-induction of ulcer. Bars carrying different letters are statistically different at $p<0.05$

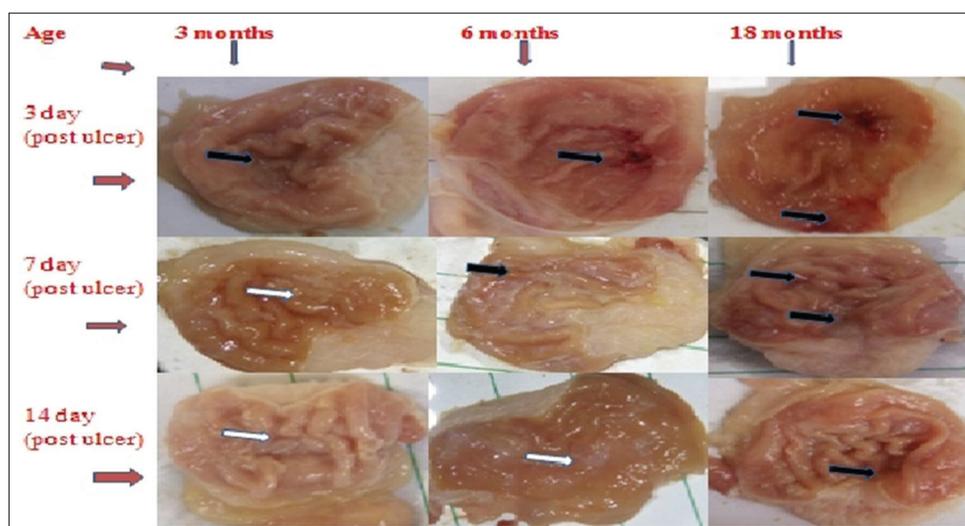


Fig. 5: Gross pictures of the stomach post-induction of gastric ulcer with acetic acid. Black arrows pointing to ulcer spots. White arrows pointing to normal mucosa

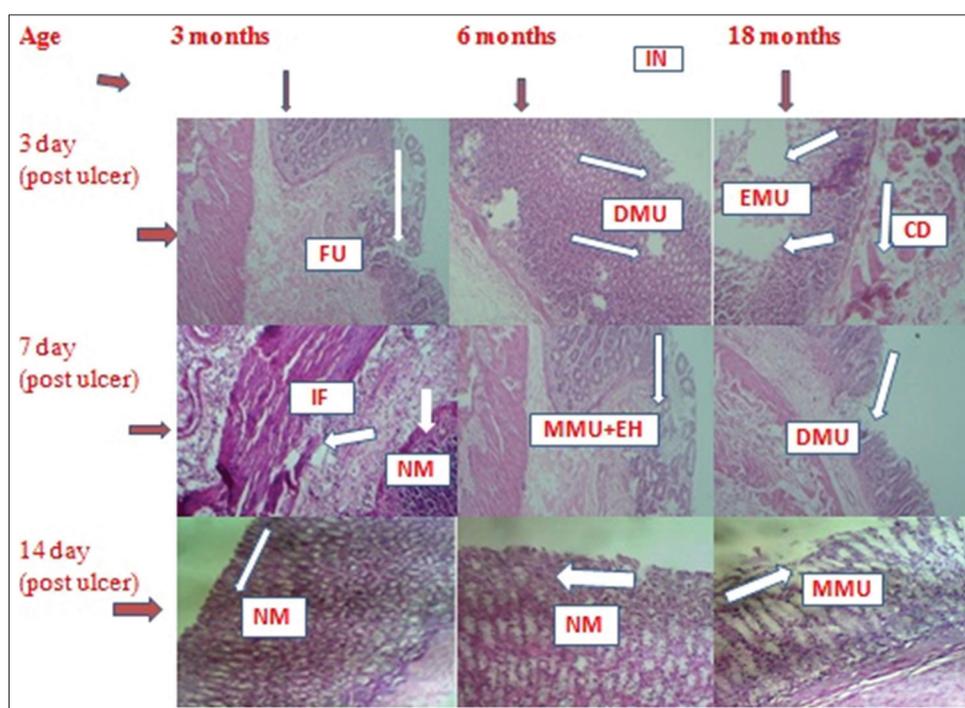


Fig. 6: Photomicrograph collage of the stomach histology of 3-, 6-, and 18-month-old rats post-acetic acid-induced ulceration. (Hematoxylin and eosin stain, $\times 100$). CD - Cellular degeneration, DMU - deep mucosa ulceration, EH - evidence of healing, EMU - extensive mucosa ulceration, FU - focal areas of ulceration, IF - infiltration of sub mucosa and muscular wall by inflammatory cells, MMU - mild mucosa ulceration, NM - normal mucosa

by Ajayi and Olaleye [20] where the rate of healing of gastric ulcer was inversely proportional to the age of rats.

Furthermore, the concentrations of PgE_2 were increased in the gastric mucosa homogenate of younger rats and decreased in that of aging rats. Pg is one of the important defensive factors protecting the gastric mucosa from damage by maintaining a balance of mucosal integrity and blood flow, which produce a healing effect [32,33]. Results from this study are similar to previous observations reported by Grønbech and Lacy [34]; they found that aging rats had decreased mucosal release of PgE_2 compared to young rats. This study, therefore, took a step forward to show that this reduced PgE_2 may delay gastric ulcer healing in the older rat by resultant decreased VEGF concentration and expression since PgE_2 can modulate the expression of VEGF [34].

NO concentrations were also significantly increased in gastric mucosa homogenate of younger rats and decreased in that of aging rats. NO synthesis has been reported to aid in the healing of gastric ulcer through increased mucosal blood flow, thus promoting angiogenesis through VEGF stimulation [35,36]. The increased NO concentration that was observed in the younger rats may be the reason for the faster healing rate observed in younger rats as a result of promoting angiogenesis.

Immunohistochemical analysis showed increased expression of VEGF in the 3- and 6-month-old rats' gastric mucosa and reduced expression in the 18-month-old rats' gastric mucosa. In addition, increases in the expression of VEGF in younger rats could have been mediated by the increased concentrations of NO and PgE_2 . Ferrara [37] had earlier

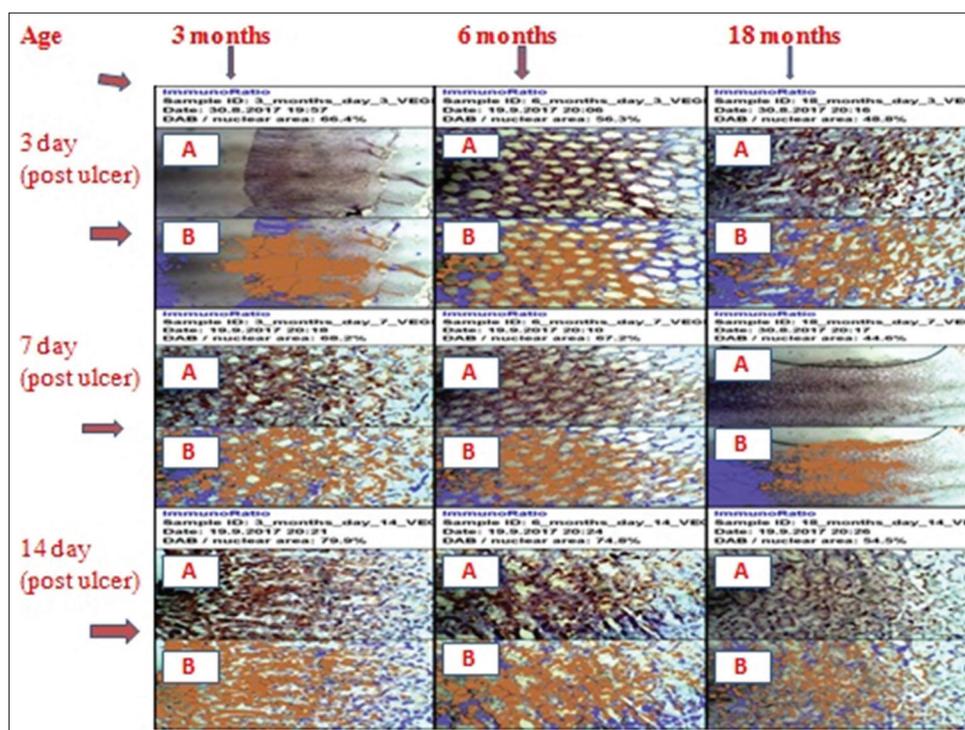


Fig. 7: Photomicrograph of expression of vascular endothelial growth factor on days 3, 7, and 14 post-induction of ulcer. A - Indicates slide of gastric mucosa with original diaminobenzidine-stained image. B - Indicates slide of gastric mucosa with a pseudo-color image produced by immunoratio web application showing staining components from which labeling index was generated

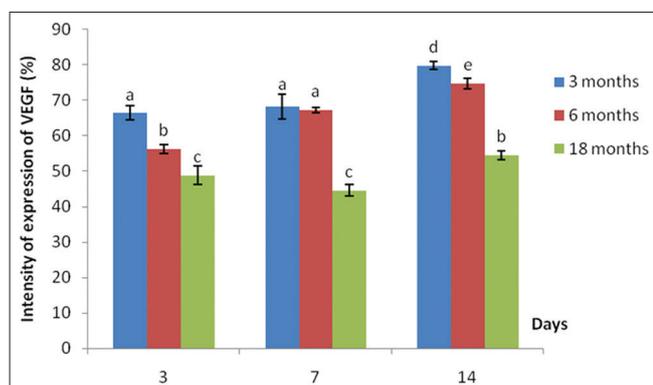


Fig. 8: Percentage intensity of the expression of vascular endothelial growth factor post-induction of gastric ulcer. Bars carrying different letters are statistically different at $p < 0.05$

reported that factors which stimulate the production of VEGF include NO and PGE_2 . These events may have led to increased VEGF expression that was observed, and this may have induced the faster healing rate observed in the younger rats.

The histology sections of the gastric mucosa confirmed the results of ulcer area and area of ulcer healed, and the faster rate of healing was observed in 3- and 6-month-old rats than 18-month-old rats. This is inconsonant with the study by Ajayi and Olaleye [20]. The delayed healing in older rats (18-month old) as displayed by the histology section of gastric mucosa could be as a result of the reduced gastroprotective potential of aging gastric mucosa as seen in the concentration of PGE_2 and NO [38], with decreased angiogenesis as displayed by VEGF concentration and expression.

CONCLUSION

The healing of ulcers in the aging gastric mucosa may be impaired due to reduced gastroprotection, epithelial restitution, and angiogenesis,

while some cellular and molecular factors in response to this have been identified such as decreased concentrations of NO, PGE_2 , and expression of VEGF. The other basic triggers for these events need further investigations since the effect of age on gastric ulcer healing may involve other body tissues apart from the gastric mucosa.

AUTHORS' CONTRIBUTIONS

Ajayi AF was responsible for the conception, design, drafting of the manuscript, and final approval of the submitted work. Kehinde BD contributed substantially to the analysis of data and the interpretation, and Lateef OM was involved in breeding of animal, induction of ulcer, and contributed to the intellectual content. Akorede BA was also involved with the breeding of animal and contributed substantially to the interpretation of data and the write up. All authors approved the submission of this article.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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