Age-related Changes in the Expression of Heat Shock Protein 70 and 90 on the Gastric Mucosa During Gastric Ulcer Healing

Ajayi Ayodeji Folorunsho¹, Aniviye Blessing Oluwafunke¹, Kehinde Busuyi David², Akintola Adebola Olayemi³

¹Department of Physiology, Ladoke Akintola University of Technology, P.M.B. 4000, Ogbomoso, Nigeria
²Department of Biochemistry, Ladoke Akintola University of Technology, P.M.B. 4000, Ogbomosho, Nigeria
³Department of Science Laboratory Technology, Ladoke Akintola University of Technology, P.M.B. 4000, Ogbomoso, Nigeria

Abstract

Heat shock proteins 70 and 90 (HSP-70 and HSP-90) are associated with gastroprotective and ulcer healing potentials. Reports in literatures have shown that age affects gastric ulcer healing, but the role of these heat shock proteins in relation to age has not been fully understood. This study, therefore, investigated changes in the expression of HSP-70 and HSP-90 in the gastric mucosa of 3, 6 and 18-month-old rats during healing of Acetic acid-induced gastric ulcers. Male Wister rats (aged 3, 6 and 18 months) were divided into 3 groups according to their ages. Acetic acid ulcer model was used for this study. Ulcer area, oxidative stress, antioxidant markers, HSP-70 and HSP-90 concentration by ELISA and expression by immunohistochemistry was assessed. Results obtained indicate the highest percentage area healed on day 14 in 3 months old rats (100%), while percentage healing for 6 and 18 months old rats was 89.00% and 55.29%, respectively. Malondialdehyde (MDA) concentration was directly proportional to age, while antioxidant enzyme (Superoxide dismutase (SOD) and catalase (CAT)) activities were inversely proportional to age. The concentration and expression of HSP-70 were inversely proportional to age while HSP-90 had directly proportionality to age. The histological architecture also confirmed the faster rate of healing in 3-month old rats recorded in this study. This study indicates that HSP-70 and HSP-90 play different roles in age-related healing of gastric ulcers.

Keywords: Ulcer, Heat shock proteins, Oxidative stress, Ulcer healing, Immunohistochemistry

1 Introduction

Studies have shown that age plays a critical role in the spontaneous healing of gastric ulcers¹; mechanisms that are, however, underlying age-related differences during healing have not been fully elucidated. The protective role of Heat Shock Proteins (HSPs) in the gastric tissues has been emphasized, but how this influence spontaneous healing of age-related gastric ulcers is not clearly known. HSPs function as molecular chaperones important in the biosynthesis, folding and unfolding, transport and assembly of cellular proteins, and also protect incorrectly folded proteins against aggregation, and facilitate the refolding of misfolded proteins². They are expressed or produced through heat shock factor transcriptional action in response to heavy metal, chemical agents, glucose, starvation, heat, oxidative and physio-pathological stresses in many organisms³⁴. HSPs are classified based on biological functions and molecular weight into HSP-90, HSP-70, HSP-60, and smaller ones like HSP-27. The type of HSP produced, and its level of expression can regulate the way a cell responds to stress or stimulus; in the gastrointestinal tract, HSPs may play a cytoprotective role⁵ and inhibit stress ulcer formation⁶. Another study showed that expression of HSP-70 corresponds with the ability of the gastric mucosa to resist mucosal damage after repeated administration of aspirin⁷. Tsukimi and Okabe⁸ observed high level of HSP-70 at the base and margin of ulcer two weeks into healing, and they suggest that this HSP is responsible for gastric mucosa regeneration.
HSPs are relevant in the composition and control of a life circle in various species and are produced as a result of both intrinsic and extrinsic stressors; they also act as the major intermediary in organism resistance to stress which corresponds with lifespan\textsuperscript{11}. Increased oxidative stress has been identified as an important mediator of HSP production during ageing\textsuperscript{9,12}. Stress can also be inhibited in old cells by increased production of HSP-70 and other HSPs, which result from the inhibition of HSF (Heat Shock Factor) together with a heat shock response through a feedback loop\textsuperscript{13}. Inhibitors of HSP-90 when administered to mouse and human cells promote health span and delayed the onset of many age-related symptoms\textsuperscript{14}. Since age plays an important role in the spontaneous healing of gastric ulcer induced with Acetic acid\textsuperscript{1,7}, it is, therefore, thought that HSPs may have essential functions in the survival and healing of mucosal cells in such states. Hence, this study focused on the expression of HSP-70 and HSP-90 and how their cytoprotective functions mediate age-related gastric mucosa ulcer healing.

2 Materials and Methods

2.1 Experimental animals

Forty-five male Wister rats of ages 3, 6 and 18 months were used for this study. The male rats were obtained from the Institute for Advanced Medical Research and Training, University College Hospital, Ibadan, Oyo state. They were acclimatized for two weeks under standard conditions (37 °C) in the Animal house of Department of Physiology, Ladoke Akintola University of Technology, Ogbomoso, Oyo state, Nigeria. The animals were kept in wire-meshed cages and fed with standard laboratory pellet diet and water ad libitum. Animals were subjected to the humane care and all procedures were conducted in accordance to the guiding principles on research involving animals recommended by the declaration of Helsinki and the guiding concepts in the care and use of experimental animals\textsuperscript{15}. 

2.2 Ethical approval

The ethical approval clearance for the implementation of the research obtained from the Oyo state research ethical review committee, Ministry of Health secretariat Ibadan, Nigeria was given a reference number: AD 13/479/459.

2.3 Experimental design

The forty-five male Wister rats were grouped into three groups according to their ages (3, 6 and 18 months) and samples were collected on days 3, 7, 14 post-ulcer induction with Acetic acid.

2.4 Ulcer induction

Gastric ulcer induction was by type 4 Acetic acid ulcer model previously described by Susumu and Kikuko\textsuperscript{16} with slight modification. Briefly, animals were fasted for 18 hours prior ulcer induction, after which the rat’s stomach was exposed under anaesthesia comprising of a mixture of 5% ketamine (35.0 mg/kg) and 2% xylazine (5.0 mg/kg) injected intramuscularly. Laparotomy was performed through a midline incision. The anterior and posterior walls of the gastric corpus were clamped together with an eye forceps and 0.2 ml of acetic acid solution (40% v/v distilled water) was injected into the clamped portion of the stomach and then withdrawn after 45 seconds. The stomach was cleaned with normal saline before the abdomen was then sutured and rats were allowed to recover before returning them to their cages having access to feed and water.

2.5 Ulcer area estimation

Gastric ulcer areas in mm\textsuperscript{2} were determined on days 3, 7 and 14 post-ulcer inductions. Rats were sacrificed by cervical dislocation on each day; stomach of each rat was removed, opened along the greater curvature, rinsed with normal saline, spread out and pinned on a cork board. The macroscopic ulcer area was measured with the aid of a 2X magnification hand lens, then measured and calculated using the collection of guiding principles of Drug administration of The Ministry of Health Beijing, 1993\textsuperscript{17} with the formula below:

\[ S = \pi \frac{(d_1/2) \times (d_2/2)}{2} \times 100 \]

\[ \text{Percentage area healed on day 7} = \frac{\text{Area of an ulcer on day 3} - \text{the area of an ulcer on day 7}}{\text{Area of an ulcer on day 3}} \times 100 \]

\[ \text{Percentage area healed on day 14} = \frac{\text{Area of an ulcer on day 3} - \text{the area of an ulcer on day 14}}{\text{Area of an ulcer on day 3}} \times 100 \]

2.6 Preparation of stomach homogenate

The stomach homogenate was prepared according to the method described by Saheed et al\textsuperscript{3}. Briefly, immediately after ulcer scoring, a fraction of the glandular portion of the stomach was cut for histological study and preserved in 10% formalin while 0.5 g each of the remaining fractions of the gastric tissue was weighed and homogenized in ice-cold 0.1 M phosphate saline buffer (1:4 w/v), pH 7.4 and the homogenates centrifuged at 2500 rpm for 10 min at 4°C. The resulting supernatants were used for biochemical analysis.

2.7 Biochemical procedures for the assay of oxidative stress indices

2.7.1 Superoxide dismutase (SOD) activity
SOD activity was measured according to the methods described by previous authors\(^{20,21}\). In this assay, the photochemical diminution of riboflavin produces O\(_2\) that reduces the nitro-blue tetrazolium (NBT) to produce formazan salt, which absorbs at a wavelength of 560 nm.

### 2.7.2 Catalase activity

Catalase activity measurement was based on the method described by earlier authors\(^{22,21}\). The rate of decomposition of H\(_2\)O\(_2\) was measured by the corresponding changes in the absorbance levels at 240 nm.

### 2.7.3 Malondialdehyde (MDA)

Assessment of lipid peroxidation was carried out by following the procedure previously described\(^{23,21}\). Reaction of malondialdehyde (MDA) generated as a result of lipid peroxidation with thiobarbituric acid (TBA) giving a pink coloured MDA-TBA that absorbs at 532 nm.

### 2.8 Concentrations of HSP-70 and HSP-90 by ELISA method

ELISA kits used in this study were obtained from Elabscience, China and the concentration of HSP-70 and HSP-90 were examined by following the manufacturer’s protocol. The concentrations of HSP-70 and HSP-90 in the samples were gotten by comparing the optical density (OD) of the samples to the standard curve.

### 2.9 Histological Study

The histology of the stomach was carried out following the procedure previously documented\(^{24,26}\). A portion of the stomach was fixed in 10% formal saline for histological study. The stomach sections were processed and embedded in paraffin wax. Then cut into 5µm sections and stained with haematoxylin and eosin (H&E). The stained sections were examined for morphological changes under a light microscope.

### 2.10 Immunohistochemistry of HSP-70 and HSP-90

Expression of HSP-70 and HSP-90 in gastric mucosa cells was determined by immunohistochemistry using anti-rat HSP-70 and HSP-90 antibodies in the presence of Streptavidin peroxidase as previously described by Gillett et al\(^{27}\).

### 2.11 Labelling index calculation from immuno ratio web application

The percentages of positively stained nuclei for HSP-70 and HSP-90 were quantified using ImmunoRatio web application (http://jsmicroscope.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, its main features include separating diaminobenzidine-stained (DAB) from haematoxylin-stained regions of the image, calculating the percentage of DAB-stained region over a total region, which is known as the labelling index and generating a pseudo-coloured image corresponding with the area segmentation\(^{28}\).

### 2.12 Statistical analysis

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

### 3 Results

#### 3.1 Age-related changes in the macroscopic ulcer dimension following induction of ulcer with acetic acid

Ulcer area (Fig 1) was significantly (p<0.05) decreased on days 7 and 14 compared with day 3 post induction of ulcer in all age groups; 3-month old rats had the least ulcer area on each day compared to 6 and 18-month old rats, while 18-month old rats had the highest ulcer area on each day compared to 6 and 3-month old rats.

![Fig 1: Age-related changes in ulcer area (mm\(^2\)) during the healing of gastric ulcer induced with Acetic acid; Bars carrying different letters are statistically different at p<0.05](http://example.com/fig1)

Figure 2 shows that the percentage ulcer area healed on days 7 and 14 decreased with advancing age during the spontaneous healing. The percentage of ulcer areas healed on day 14 were 100%, 89% and 55.29% in 3, 6, and 18-month old rats, respectively.

![Fig 2: Age-related changes in the percentage area healed on day 7 and day 14 of gastric ulcer healing; Bars carrying different letters are statistically different at p<0.05](http://example.com/fig2)
3.2 Effect of age on oxidative stress indices

Table 1 shows the age-related changes in the activities of antioxidant enzymes; Superoxide dismutase (SOD), and Catalase (CAT), and a lipid peroxidation marker; Malondialdehyde (MDA) during gastric ulcer healing. Superoxide dismutase and Catalase activities increased significantly (p<0.05) on days 7 and 14 when compared with day 3 in all age groups. The activities of these antioxidant enzymes were significantly (p<0.05) higher in the three months old rats when compared with 6 and 18-month old rats.

Table 1: Superoxide dismutase (SOD), Catalase (CAT) and Malondialdehyde (MDA) levels after ulcer induction

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>DAY 3</th>
<th>DAY 7</th>
<th>DAY 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (µmol/min)</td>
<td>3 months</td>
<td>3.98 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.16 ± 0.74&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6 months</td>
<td>3.49 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.95 ± 0.61&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>18 months</td>
<td>1.97 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.78 ± 0.33&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT (µmol/min)</td>
<td>3 months</td>
<td>23.02 ± 0.74&lt;sup&gt;d&lt;/sup&gt;</td>
<td>32.42 ± 0.86&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6 months</td>
<td>18.94 ± 0.43&lt;sup&gt;d&lt;/sup&gt;</td>
<td>24.52 ± 0.92&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>18 months</td>
<td>16.64 ± 0.19&lt;sup&gt;d&lt;/sup&gt;</td>
<td>23.78 ± 1.30&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>MDA (nmol/g)</td>
<td>3 months</td>
<td>14.26 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.76 ± 0.40&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6 months</td>
<td>16.68 ± 0.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.45 ± 0.76&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>18 months</td>
<td>19.60 ± 0.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.66 ± 0.92&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Legend: Superscripts a, b, c, d, e, f, g= different letters are showing that Figures are statistically different at p<0.05. Malondialdehyde (MDA) concentration was significantly (p<0.05) higher in 18-month old rats compared to 6 and 3-month old rats post induction of gastric ulcer with acetic acid.

3.3 Effects of age on the concentration of HSP-70 and HSP-90 in the gastric tissue homogenate post induction of ulcer

Concentration of HSP-70 in gastric tissue homogenate (Fig 3) significantly increased during gastric ulcer healing in all age groups; 3-month old rats had a significantly (p<0.05) higher concentration than the 6 and 18-month old rats on days 3 and 7, but the difference between the concentrations of 3 and 6-month old rats on day 14 was not statistically significant (p>0.05).

The concentration of HSP-90 was higher on day 3 post induction of ulcer compared with the concentrations on days 7 and 14 after gastric ulcer induction, also, the 18 months old rats had significantly higher concentration of HSP-90 compared with the concentrations of HSP-90 in 3 and 6 months old rats (Fig 4).

Fig 4: Age-related changes in HSP-90 concentration in gastric tissue homogenate during healing of gastric ulcer induced with Acetic acid.; Bars carrying different letters are statistically different at p<0.05

3.4 Collage of the rats’ stomach gross pictures post induction of gastric ulcer with Acetic acid

Figure 5 shows gross pictures of rats’ stomach on days 3, 7 and 14 post Acetic acid ulcer inductions. Eighteen (18)-month old rats had more haemorrhagic streaks and spot ulcers when compared with 3 and 6-month old rats 3 days post induction of ulcer. By day 14, normal gastric mucosa with mucus coating was seen in 3-month old rats, while, near normal gastric mucosa with mucus coating was observed in 6-month old rats but 18-month old rats showed gastric mucosa with mild ulcerated spots.
3.5 Collage of stomach sections of rats' histology at different ages during gastric ulcer healing

Figure 6 shows the age-related changes in the architecture of gastric tissue on days 3, 7 and 14 post Acetic acid inductions.

3.6 Immunohistochemical expression of HSP-70 post Acetic acid ulceration

Figures 7 and 8 are showing the expression of HSP-70 and its intensity, respectively following immunohistochemical staining post Acetic acid ulceration. On day 3, the labelling index of 59.5%, 40.4% and 38.7% were obtained for 3, 6 and 18-month old rats, respectively. On day 7, the labelling index of 77%, 49% and 44% were obtained for 3, 6 and 18-month old rats, respectively. On day 14, the labelling index of 78.2%, 59.2% and 56.4% were obtained for 3, 6 and 18-month old rats, respectively.

3.7 Immunohistochemical Expression of HSP-90 post with Acetic acid ulceration
Figure 9 and Figure 10 shows expression of HSP-90 and its intensity, respectively following immunohistochemical staining post ulcer induction. On day 3, labelling index of 52.2%, 58.7% and 87.2% were obtained for 3, 6 and 18 months old rats, respectively. On day 7, labelling index of 48.5%, 56.9% and 83.2% were obtained for 3, 6 and 18 months old rats, respectively. On day 14, labelling index of 41.2%, 42.7% and 67.2% were obtained for 3, 6 and 18 months old rats, respectively.

Figure 7: Collage of photomicrographs showing expression of HSP-70 on days 3, 7 and 14 post Acetic acid ulceration; Legend: A = Indicates slide of gastric mucosa with original DAB-stained image.; B = Indicates slide of gastric mucosa with a pseudo-coloured image produced by ImmunoRatio web application showing staining components from which labelling index was generated.

Fig 8: Intensity of HSP-70 expressions in the gastric tissue homogenate of rats following Acetic acid induced ulcer in rats of different ages.; Legend: Bars carrying different letters are statistically different at p<0.05

4 Discussions

The ulcer model used resembles human ulcers in pathological features and healing mechanisms as reported by previous authors. Formation of ulcer was established in this study as evidenced by the higher percentage of ulcer area on day 3 while healing was ascertained by the increase in the area of ulcers healed. The highest area of ulcer healed was obtained in the 3-month old rats, and we observed that healing was inversely proportional to rats’ age. Ajayi and Olaleye had previously reported that gastric ulcers healed faster in younger rats than in older rats. Ageing has been indicated in the delay of ulcer healing due to increased susceptibility to aggressive factors and reduction in protective physiological factors.

Healing of gastric ulcer involves restoration of the balance between the aggressive and defensive factors through a genetically programmed process involving inflammation, cell proliferation, re-epithelisation, development of granulation tissue, the formation of blood vessels, interaction of various cells with the matrix and tissues remodelling leading to scar formation. Results from gastric tissue histology indicated faster healing in the 3 month and 6-month old rats, this was evidenced by the appearance of normal mucosa architecture on day 14, while 18-month old rats still presented with shreds of evidence of inflammation on days 7 and 14. This observation is similar to some previous studies that attributed faster healing in younger rats to inherent anti-inflammatory properties of the gastric mucosa.

Furthermore, observations from our study revealed that gastric tissue homogenate activities of SOD and Catalase increased.
significantly during ulcer healing with the 3-month old rats having higher activities of SOD and Catalase compared to 6 and 18-month old rats.

### Fig 9: Collage of photomicrographs showing expression of HSP-90 on days 3, 7 and 14 post Acetic acid ulceration. Legend: A = Indicates slide of gastric mucosa with original DAB-stained image.; B = Indicates slide of gastric mucosa with a pseudo-coloured image produced by ImmunoRatio web application showing staining components from which labelling index was generated.

### Fig 10: Intensity of HSP-90 expressions in the gastric tissue homogenate of rats following Acetic acid induced ulcer in rats of different ages. Legend: Bars carrying different letters are statistically different at p<0.05

Endogenous antioxidant enzymes like SOD and catalase are important for organisms to scavenge reactive oxygen species and reduce intracellular stress, this not only protects the gastric tissue against injury but also, play important role in healing in the case of any event of gastric inflammation or ulcers. Ajayi and Olaleye also identified the role of antioxidant enzymes and oxidative stress in age-related gastric ulcer healing; findings from their results are similar to what we also observed on oxidative stress indices in this study.

MDA a major metabolite of lipid peroxidation is an indicator of mucosal inflammation resulting from oxidative stress. In this study, we observed increased levels of MDA in 18-month old rats compared to 3-month old rats. This we also, found to be corresponding to the extent of gastric tissue damage and may suggest that lipid peroxidation is one of the factors responsible for reduced healing rate observed in the 18-month old rats compared to 3-month old rats.

HSPs are molecular chaperones that play important role in gastric defence by improving cellular integrity at intracellular levels. They conduct folding, assembly, transport of proteins and protect cells from the cytotoxic effects of aggregated proteins produced during stress. HSPs achieved effective gastric mucosal protection process and promote ulcer healing via important enzymes related to cytoprotection. Tsukimi and Okabe reported low HSP-70 in normal mucosa and higher in ulcer bases during ulcer formation. In conjunction to this, Soncin and Calderwood proposed that HSP-70 is expressed in proliferating cells during re-epithelialization phase of ulcer healing and may, therefore, be involved in the regeneration of ulcerated mucosa. This means that HSP-70 induction at the bases of ulcer will either produce new proteins or regulate the function of important enzymes in ulcer healing by its own molecular chaperone property.
Results from this study indicate that HSP-70 was markedly expressed in the ulcer base on day 3 post ulcer induction. This confirms its expression in proliferating cells and thus, its involvement in re-epithelialization and mucosal regeneration. Thus, increased localization of HSP-70 in the ulcer base of gastric tissue and its increased expression during ulcer healing may have contributed significantly to faster ulcer healing observed in the 3-month old rats.

Our study also, found that the expression of HSP-90 was significantly higher in 18-month old rats while 3-month old rats had lower expressions. Wei et al. reported that HSP-90 enables cells to cope with environmental changes such as tissue injury, but that tumour cells take advantage of it for metastasis. We are however, not sure of why higher expressions of HSP-90 was seen in 18-month old rats and lower expressions in 3-month old rats but may indicate that HSP-90 has some pro-inflammatory properties. Previous authors had reported that HSP-90 may facilitate cell damage and promote carcinogenesis.

5 Conclusions

In conclusion, our findings from this study indicate that HSP-70 and HSP-90 have different relationship with age during the healing of gastric ulcers; the expression of HSP-70 is inversely proportional to age, thereby, promoting accelerated gastric ulcer processes in 3-month old rats. While, the expression of HSP-90 is directly proportional to age and may contribute to the delayed healing process in 6 and 18-month old rats which needs further investigations.

6 Conflicts of interest

The authors declare that there are no conflicts of interest.

7 Author contributions statement

AAF: Conceptualized and designed the studies, AAO, ABO and KBD, performed the experiments, KBD, ABO and AAO. Contributed materials and analysis tools, AAF and ABO, drafted and wrote the final manuscript.

8 References

17. Salami AT, Ndukauba, NG, Iyiola, TO, Agbola OF, Oluwole FS, Olaleye SB. Gastroprotective Properties of Manganese Chloride on Acetic Acid Induced Ulcer. UK J Pharm & Biosci, 2018; 6(4); 8


39. Fornai M, Antonioli L, Colucci R, Tuucci M, Blandizzi C. Pathophysiology of Gastric Ulcer Development and Healing: Molecular Mechanisms and Novel
Therapeutic Options, Peptic Ulcer Disease Jianyuan Chai, Intech.Open, 2011; DOI: 10.5772/17640.
