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Original Article

Therapeutic effect of silver/chitosan/ascorbic acid nanocomposites on ethanol-induced gastric ulcers in rats

Andrew Malak[1], Nagat Saleh Hossuin Moftha[2], Emad M. El-Zaya[3], Ayman S. Mohamed[3,*] and Dalia Y. Saad[3]

[1]Faculty of Biotechnology, October University for Modern Sciences and Arts (MSA), Intersection of 26th of July with Al Wahat Road, Giza, Egypt. [2]Zoology Department, Faculty of Arts and Sciences Alabyar, University of Benghazi, Uruba Street, Bangazi, Libya. [3]Zoology Department, Faculty of Science, Cairo University, Gamaa Street, Giza, Egypt.

ABSTRACT

The present study aimed to utilize all the benefits of silver, chitosan, and ascorbic acid -in the form of nanoparticles- such as wound healing, antioxidant, antibacterial, and anti-inflammatory properties in treating gastric ulcers. Thirty rats were assigned into five groups. Gastric ulcer was induced by 99% ethanol (5 ml/kg body weight; orally). One hour later, the ulcerated rats were administrated distilled water (Ulcer group), 0.25 mg/Kg of silver-chitosan-ascorbic acid nanocomposites (NC group), 50 mg/Kg of ranitidine (Ran. group), and NCs + ranitidine (NC + Ran. group). NCs and or ranitidine caused a significant decrease in ulcer index, gastric juice volume, and malondialdehyde, while gastric juice pH, glutathione, and catalase increased significantly in addition to the significant histological improvement of the gastric mucosa. Silver-chitosan-ascorbic acid nanocomposites are a promising treatment modality against gastric injury through their powerful antioxidant, acid neutralizing, and healing promotion effects.

KEYWORDS: silver, chitosan, ascorbic acid, nanocomposite, ulcer, oxidative stress.

1. Introduction

Peptic ulcer is one of the most prevalent diseases of the alimentary tract. It affects around 4 million of the total population yearly, with nearly 10-20% complications [1]. It occurs due to damage of the gastro-duodenal mucosa, leading to exposure of the underlying layers to the digestive action of various alimentary tract secretions [2]. Depending on the lesion site, the peptic ulcer could be either gastric or duodenal [2, 3]. The incidence of gastric ulcers reaches about 10% worldwide, with an annual rise in prevalence [4].

Ethanol is a well-known destructive agent to the gastric mucosa [5], causing edema, erosions, ulcerative lesions, hemorrhage, and encouraging infiltration of inflammatory cells, the release of free radicals, and oxidative stress [6].

Current anti-ulcer drugs could be classified into gastric acid secretion inhibitors, proton pump inhibitors, and *H. pylori* growth inhibitors [7]. Despite these various treatment options, ulcer complications can still occur, besides the numerous drug adverse effects [8]. Therefore, the search for a new anti-ulcer treatment for gastric ulcers with fewer side effects and higher compliance than current treatments is required [9].

Chitosan polymer is a natural polysaccharide prepared by chitin deacetylation [10]. It is a naturally abundant biodegradable polymer material used in the biomedicine field because of its antibacterial, anti-inflammatory, anti-thrombotic, hemostatic, wound healing, and immune modulation activities [11].

*Corresponding author: ayman81125@cu.edu.eg

One of the most promising nano-materials is silver NC (Ag-NCs) due to its antimicrobial [12], anti-inflammatory [13], antifungal [14], wound healing [15], and antiviral activities [16]. Besides, a previous study recorded the efficiency of Ag-NCs in enhancing ulcer healing [17].

Ascorbic acid is a potent antioxidant molecule that plays a tremendous cytoprotective role against oxidative damage involved in the progress of different diseases [18]. Furthermore, ascorbic acid plays a significant role in synthesizing collagen and wound healing [19]. Owing to all these beneficial actions of Ag, chitosan, and ascorbic acid, we hypothesized that combining them as one compound in the form of nanoparticles may yield a powerful antiulcerogenic agent.

2. Materials and methods

2.1. Chemicals

Medium molecular weight chitosan (1278 ± 10 KDa) with varying deacetylation degree (> 80%), acetic acid, silver nitrate ($AgNO_3$ ≥ 99%), ascorbic acid (≥ 99%), and sodium hydroxide (≥ 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Biochemical Kits were purchased from Bio-diagnostic Company (Dokki, Giza, Egypt).

2.2. Silver-chitosan-ascorbic acid NC synthesis

Chitosan-based silver NCs were synthesized according to the method of Regiel-Futyra [20]. One gram of chitosan was added to 100 ml of 1% acetic acid and then heated up to 95 ^{o}C using an oil bath. Next, 7 mM of $AgNO_3$ and 1 mM of ascorbic acid solutions were added drop-wise in the following volume ratio: chitosan: $AgNO_3$: ascorbic acid, 100: 20: 20, respectively. This mixture was kept under heating and stirring for 12 hrs. It was then dried in an electric oven (Pol-Eko) at 60 ^{o}C until the solvent's complete evaporation. Lastly, the mixture was neutralized with 1% NaOH solution, washed with deionized water, dried, and kept in the dark until further use.

2.3. Characterization of Ag-NCs

The formation of Ag-NCs was confirmed by UV-Vis absorption spectra. The reduction of the Ag^{+} ions in solution was observed by periodic sampling of the reaction mixture, and the maximum light absorption of the Ag-NCs was scanned by UV-Vis. A spectrophotometer (Shimadzu UV-1601) at a wavelength range of 200-700 nm and operated at an interval of 10 nm was used during the analysis.

A Bruker D2 diffractometer was used to analyze the X-ray Diffraction (XRD) patterns at 40 kV and 50 mA. The secondary graphite monochromated Co K alpha radiation (l = 1.7902A˚) was used, and the measurements were recorded at a high angle 2 theta (2θ) at a range of 5˚-90˚ with a scan speed of 0.01°.

The transmission electron microscopy (TEM) technique was used to detect the morphology and the size of the synthesized Ag-NCs. A JEOL JEM-2100 transmission electron microscope operating at 200 kV was used. TEM grids were prepared by placing a drop of the particle solution on a carbon-coated copper grid and drying it under the lamp.

2.4. Hydrogen peroxide scavenging (H_2O_2) assay

A hydrogen peroxide solution (40 mM) was prepared in a phosphate buffer (50 mM, pH: 7.4). The concentration of hydrogen peroxide was determined by absorption at 240 nm using a spectrophotometer [21]. Graded concentrations of the NCs (10-60 µg/mL) in distilled water were added to H_2O_2 and absorbance at 240 nm was determined after 1 min against a blank solution containing phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging was calculated as follows:

$$\% \text{ scavenged } H_2O_2 = \frac{A1 - A2}{A1} \times 100,$$

where A1 is the absorbance of control and A2 is the absorbance of the NCs.

2.5. Animals

Thirty male Albino Wistar rats (*Rattus norvegicus*) weighing 180 ± 10 gm were obtained from the National Research Center (NRC) and used in this study. They were housed in a well-ventilated room with temperature 22 to 25 °C and maintained under a 12:12 hrs light:dark cycle. Rats were grouped randomly (six per cage) and

housed in static polycarbonate plastic cages (height: 20 cm and floor space: 860 cm^2). Food, not water, was withdrawn 24 hrs before the experiment began.

2.6. Ethics approval and consent to participate

This study's experimental protocols and procedures were approved by Institutional Animal Care and Use Committee (IACUC) in accordance with the international guidelines for care and use of laboratory animals.

2.7. Experimental design

The animals were randomly divided into five groups (6/group):

Control group: rats administered distilled water (5 ml/kg body weight; orally), followed by distilled water one hour later.

Ulcer group: rats administered 99% ethanol (5 ml/kg body weight; orally), followed by distilled water one hour later.

NC group: rats administered 99% ethanol (5 ml/kg body weight; orally), followed by NCs (0.25 mg/Kg body weight; orally) one hour later [22].

Ran. group: rats administered 99% ethanol (5 ml/kg body weight; orally), followed by ranitidine (50 mg/Kg body weight; orally) one hour later [23].

NC + Ran. group: rats administered 99% ethanol (5 ml/kg body weight; orally), followed by administration of both NCs (0.25 mg/Kg body weight; orally) and ranitidine (50 mg/Kg body weight; orally) one hour later.

Twenty-four hrs later, all the rats were euthanized by a lethal dose of sodium phenobarbital (100 mg/Kg; intraperitoneal injection). The stomach was dissected, and the gastric juice was collected. A portion of the stomach was dissected and homogenized in a cold potassium phosphate buffer (0.05 M, pH: 7.4). The homogenate was centrifuged at 5000 rpm for 10 min. The supernatant was stored at -80 °C until the measurement of oxidative stress markers.

2.8. Ulcer markers

The gastric juice collected after opening the stomachs was centrifuged at 3000 rpm for 10 minutes to remove any solid remnants and the volume of the supernatant was measured. The pH of gastric juice was determined using a pH meter. The stomachs were examined for ulcers under magnification (x10). The ulcer index was assessed as follows: if the ulcer length is: < 1.0 mm = 1 point, between 1 and 2 mm = 2 point, ≥ 3 mm = 3 point. The sum of the scores were divided by 10 (magnification of the lens) to obtain the ulcer index in rats [24].

2.9. Oxidative stress markers

The stomach supernatant was used for the determination of malondialdehyde (MDA), reduced glutathione (GSH) and catalase (CAT).

2.10. Histopathological examination

The stomach tissue was fixed in 10% neutral-buffered formalin. The fixed specimens were washed, dehydrated, and embedded in paraffin wax. The tissues were sectioned at a thickness of 4-5 μm and stained with hematoxylin and eosin (H&E).

2.11. Statistical analysis

All results were expressed as means ± standard deviation (SD). For comparison of variances between the groups, one-way analysis of variance (ANOVA) with the Duncan post hoc test was used via SPSS for Windows (version 15.0, Inc., Chicago, IL, USA) software. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Characterization of Ag-NCs

The absorption spectrum of the synthesized Ag-NCs suspension showed a surface plasmon absorption band with a peak at 425 nm, which indicates the formation of nanosized particles (Figure 1A).

The analysis of the crystalline size and structure of the Ag-NCs was carried out by XRD as shown in Figure 1B. The numbers 39.40^o, 44.11^o, 65.11^o, and 78.24^o of Bragg reflections with 2θ values indicated that the Ag-NCs were spherical and crystalline.

The TEM analyses of Ag-NCs showed that the nanoparticles had a spherical shape and the size was ranging between 6 and 25 nm (Figure 2).

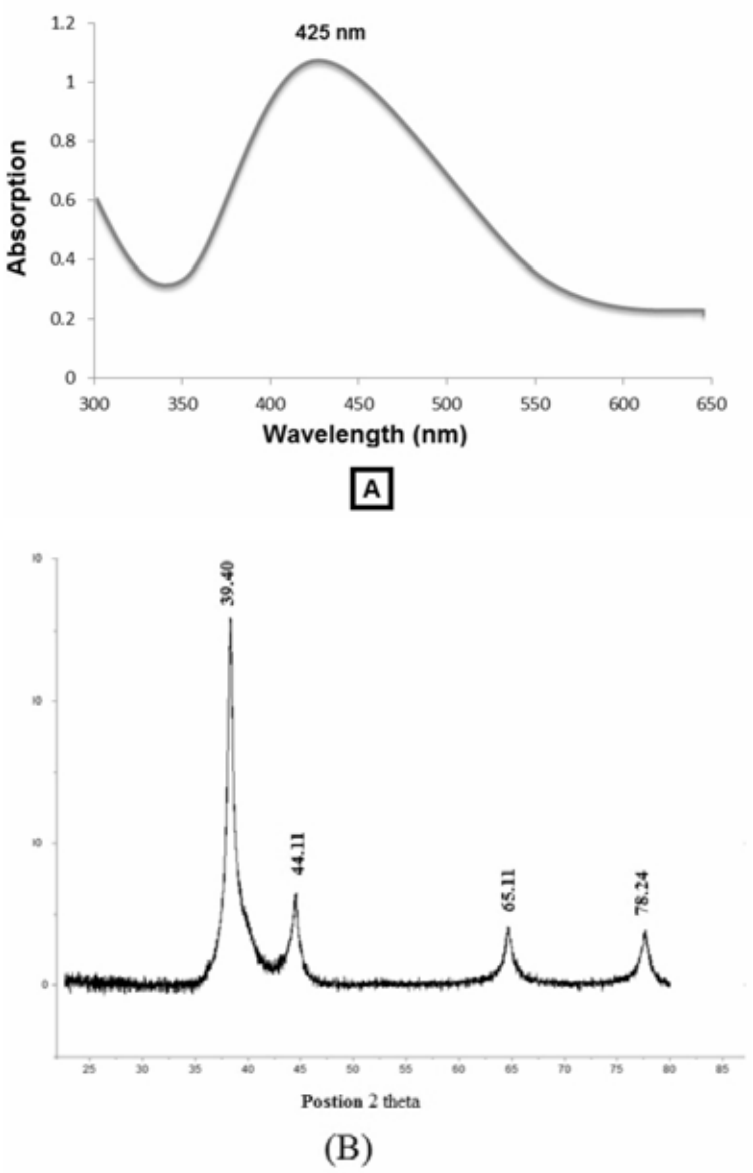

Figure 1. (A) UV-Visible spectral analysis of Ag-NCs. (B) XRD analysis of Ag-NCs.

3.2. Hydrogen peroxide scavenging (H_2O_2) assay

Hydrogen peroxide scavenging activity represents the antioxidant activity of the substances. Ag-chitosan-ascorbic acid NCs showed H_2O_2 scavenging activity, which proportionately increases with increase in the nanoparticles concentrations (Figure 3).

3.3. Ulcer parameters

The ulcer index and gastric juice volume increased significantly ($p<0.05$) in the ulcer group, while gastric juice pH decreased significantly compared with the control group. On the other hand, the treatment with NCs and/or Ran. caused a significant decrease ($p<0.05$) in ulcer index and gastric juice volume, while the gastric juice pH significantly increased as compared to the ulcer group (Table 1). The percentage of change in the parameters showed that the treatment with NCs achieved the highest outcome.

3.4. Oxidative stress markers

Data recorded in Table 2 demonstrated that ethanol caused a significant increase ($p < 0.05$) in MDA concentration. At the same time, GSH and

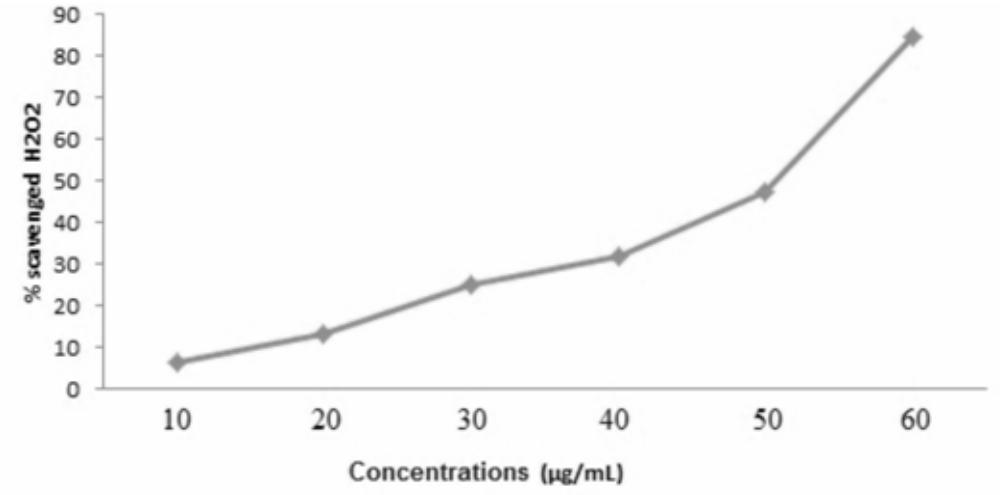

Figure 2. H_2O_2 scavenging activity of Ag-NCs.

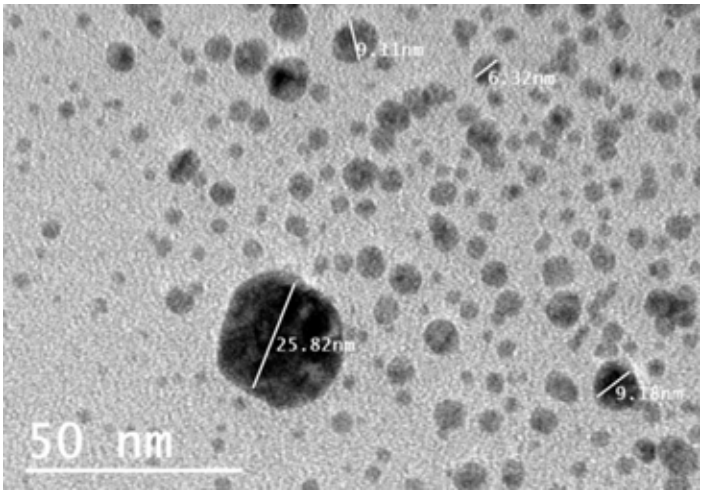

Figure 3. TEM analysis of Ag-NCs.

Table 1. Curative effect of AgNPs and/or Ranitidine on markers of gastric ulcer induced by ethanol in rats.

Parameters	Statistical values	Control	Ulcer	NC	Ran.	NC + Ran.
Ulcer index	Mean	0.10 ± 0.00^{a}	0.57 ± 0.07^{c}	0.24 ± 0.07^{b}	0.36 ± 0.02^{b}	0.28 ± 0.03^{b}
	% of change		474	140	260	175
Gastric juice pH	Mean	4.90 ± 0.13^{b}	3.48 ± 0.29^{a}	4.73 ± 0.08^{b}	6.57 ± 0.80^{c}	5.96 ± 0.35^{bc}
	% of change		- 29.01	- 3.51	34.07	21.54
Gastric juice volume (ml)	Mean	3.21 ± 0.23^{a}	5.35 ± 0.19^{c}	4.11 ± 0.18^{b}	4.53 ± 0.40^{b}	4.64 ± 0.45^{b}
	% of change		66.67	28.04	41.12	44.55

Values are mean ± SEM (n = 6). Values with different superscript letters are significantly different ($P < 0.05$).

Table 2. Curative effect of AgNPs and/or Ranitidine on oxidative stress markers of gastric ulcer induced by ethanol in rats.

Parameters	Statistical values	Control	Ulcer	NC	Ran.	NC + Ran.
MDA (nmol/g.tissue)	Mean	0.12 ± 0.00^{a}	0.22 ± 0.01^{c}	0.15 ± 0.01^{b}	0.16 ± 0.01^{b}	0.16 ± 0.01^{b}
	% of change	-	80.00	21.67	31.67	36.67
GSH (mg/g.tissue)	Mean	6.25 ± 0.13^{c}	3.60 ± 0.33^{a}	5.30 ± 0.48^{c}	5.09 ± 0.31^{b}	5.09 ± 0.31^{b}
	% of change		-42.32	-15.20	-18.57	-18.57
CAT (U/min)	Mean	5.34 ± 0.18^{c}	2.98 ± 0.23^{a}	4.54 ± 0.47^{bc}	4.11 ± 0.28^{b}	4.17 ± 0.38^{b}
	% of change		-44.19	-14.94	-23.11	-21.95

Values are mean ± SEM (n = 6). Values with different superscript letters are significantly different ($P < 0.05$).

CAT levels decreased significantly in the ulcer group compared to the control group. The treatment with NCs and/or Ran. decreased MDA concentration significantly and elevated GSH and CAT levels significantly compared to the ulcer group. The percentage of change in the parameters showed that NCs were the most effective treatment option.

3.5. Histopathological examination of the stomach

Histological examination of the control group showed normal gastric wall with intact mucosa as well as glandular structures (black star), intact submucosa (red star), and muscular coat as well as the outer serosal membrane (Figure 4A). However, the stomach of the ethanol-induced ulcer group revealed focal areas of erosions and ulcerations in the gastric mucosa with necrotic tissue debris (black star), and submucosal edema with moderate inflammatory cell infiltration (red star) (Figure 4B). On the other hand, stomachs of rats treated with NCs and/or Ran. showed intact mucosal lining without any abnormal alterations, but submucosal edema and mild inflammatory cell infiltrates (red star) were noticed (Figure 4 C, D, E).

4. Discussion

The present research was conducted to evaluate the antiulcerogenic activity of Ag-chitosan-ascorbic acid NCs versus Ranitidine against acute gastric ulceration induced by ethanol in rats. Accordingly, we assessed some ulcer and oxidant-antioxidant parameters in gastric tissues to reconnoiter their role in our experiment.

Ethanol-induced gastric ulceration provides an ideal model for evaluating the therapeutic activity of different active products [25]. In the current study, oral administration of ethanol caused acute lesions in the gastric mucosa reflected as an increased ulcer index, gastric juice volume, and MDA concentration, while it caused a significant decrease in gastric juice pH, GSH, and CAT levels with extensive damage to the gastric mucosa. The various mechanisms through which ethanol acts on the stomach include inhibition of bicarbonate secretion [26], disruption of the gastric mucosal barrier, and provocation of pronounced microvascular changes within a few minutes after its application [27]. In addition, ethanol reduces gastric blood flow and encourages oxidative stress by increasing the production of MDA and reducing the production of the antioxidants GSH and CAT [28]. Also, ethanol leads to inflammatory cell infiltration and epithelial cell loss in the stomach, causing mucosal damage and necrosis [29].

The therapeutic efficacy of the NCs was evident from the diminished ulcer index, gastric juice

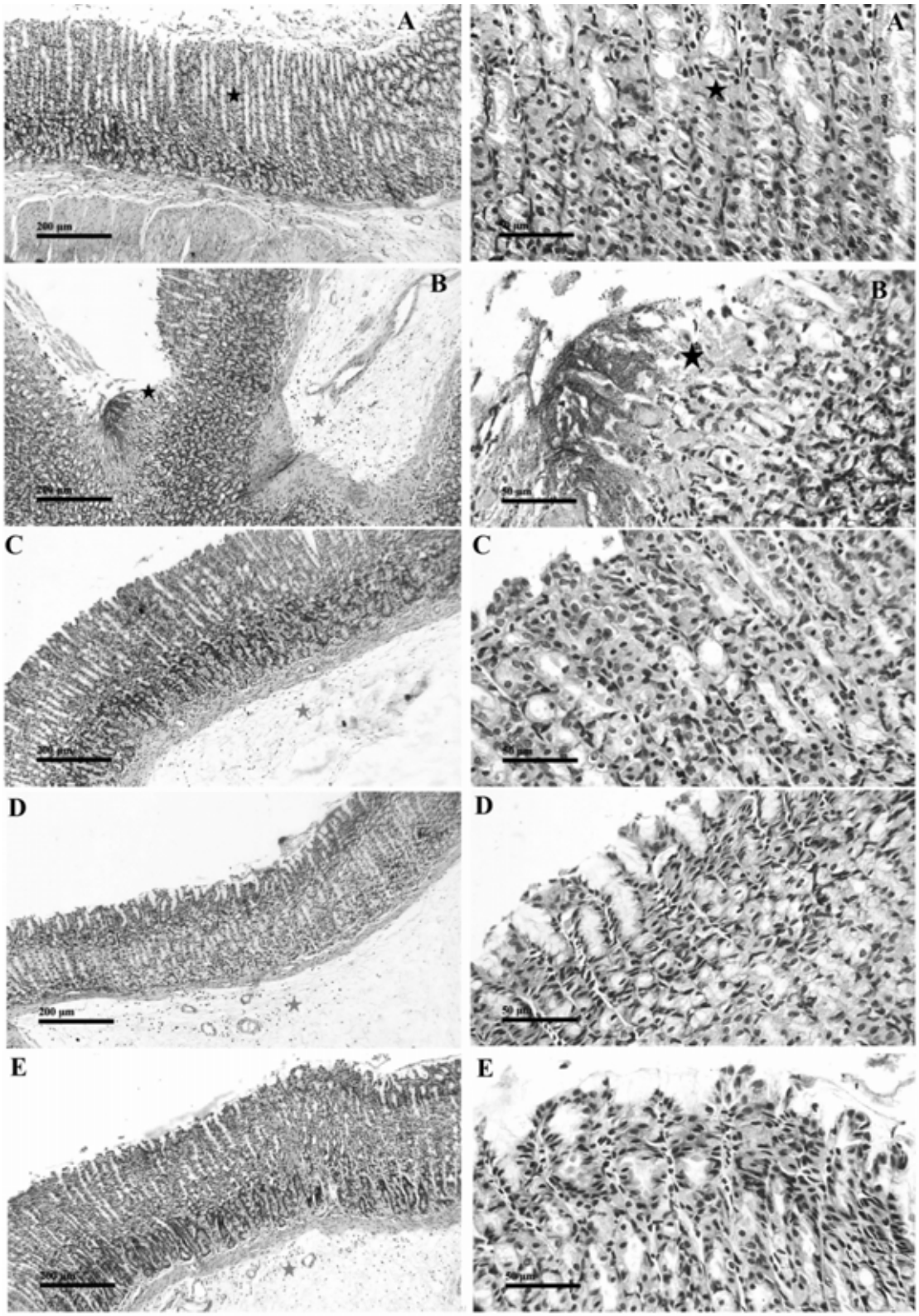

Figure 4. Histopathological examination (H & E) of stomach sections: A: Control group, B: ulcer group, C: NC group, D: Ran group and E: NC + Ran. group.

volume, and MDA concentration, and elevated gastric juice pH, GSH, and CAT levels concomitant with a significant improvement of the gastric mucosa.

This curative effect of the NCs may be due to synergism between the different compounds: Ag-nanoparticles, chitosan, and ascorbic acid, as explained below, yielding this significant effect.

Due to its multiple properties, chitosan has multiple nutritional uses. [30] Chitosan is a natural, rigid, cationic, non-toxic, biodegradable, and biocompatible polymer [31] used in wound healing due to its antimicrobial and anti-inflammatory activities [32]. In addition, Anandan *et al.* reported that chitosan had reactive oxygen species (ROS) scavenging activity [33]. The antioxidant capacity of chitosan, irrespective of the involved mechanism, is closely related to the reactivity of the active hydroxyl and amino groups in polymer chains [34]. Chitosan has been reported to trap free radicals by a non-enzymatic process of electron donation and radical resonating mechanisms, impeding lipid peroxidation chain reaction [35]. Furthermore, *Zhang et al.* reported that oral administration of chitosan improved the gastric emptying rate and had a healing promoting effect on gastric ulcer in rats *via* enhancing collagen expression and secretion by fibroblasts [36]. Furthermore, Perumcherry Raman *et al*. [30] reported that anthocyanin-loaded chitosan nanoparticles increase the expression of anti-inflammatory cytokines (Interleukin 4, IL-4) and suppress pro-inflammatory cytokines (I-nterferongamma, IFN-γ), resulting in inhibition of the inflammation associated with gastric ulcer. The increase in gastric juice pH may be due to the chitosan acid-neutralizing effect *via* the gradual release of glucosamine residues into the gastric mucosa [33]. Additionally, chitosan has antibacterial activity against *H. pylori* [37], besides its ability to form a gel in the stomach, protecting it from the digestive effect of acid and pepsin [38]; all of these actions contribute to the anti-ulcerogenic effects of chitosan.

Also, ascorbic acid plays various vital roles in the body [39]. It is known that ascorbic acid enhances antioxidant activity and inhibits lipid peroxidation [40]. In addition, ascorbic acid supplementation likely relates to inhibiting the incidence of bleeding from peptic ulcers [41]. It was reported that ascorbic acid inhibits the growth of *H. pylori in vitro* [42]. This inhibitory effect of ascorbic acid is because it is a potent antioxidant and an immune-boosting agent, an inhibitor of urease, a potent collagen synthesizing agent, and a strong stimulant of prostaglandin synthesis [43].

Such stunning effects of NCs could be strongly related to the mucoadhesive property of chitosan that prolongs the gastric residence time of the NCs [44]. These NCs are generally non-toxic, well-tolerated, and biocompatible [45]. Besides, the high permeation properties of chitosan enhance the paracellular uptake of NCs *via* the reversible opening of the tight junctions between epithelial cells and the transcellular uptake of the nanoparticles (NPs) across the epithelial cells [46]. Furthermore, unlike microparticles, the cellular uptake of NPs is much easier for immune cells, like macrophages, in the inflamed area leading to their selective accumulation in the ulcerative tissues [47].

Additionally, the NCs remarkable outcome could be attributed to their anti-inflammatory and antibacterial properties [48]. Also, the wound healing mechanism of Ag-NCs might act through the enhancement of re-epithelization process, resulting from their ability to down-regulate the inflammatory response, decrease the cytokines and matrix metalloproteinases level, and stimulate the inflammatory cell apoptosis, leading to early wound healing [49].

Surprisingly, it was expected to acquire a more powerful curative effect upon the administration of both ranitidine which is a histamine (H2) receptor blocker [38] and an antioxidant [50], and Ag- chitosan-ascorbic acid-NCs due to summation of the beneficial effects of both, but the outcomes of both were nearly the same; even more, Ag-chitosan-ascorbic acid-NCs effects were superior to those of ranitidine. Thus, it shows that Ag-CS-ascorbic acid NCs may be a better alternative to Ranitidine in gastric protection with fewer side effects, less cost, and more compliant lower doses.

5. Conclusion

The remarkable beneficial effects of Ag-chitosan-ascorbic acid NCs on ethanol-induced gastric

ulcer model may offer a promising treatment modality- being a less toxic and a cost-effective natural polymer- over the current anti-ulcer drugs, which have been accused of giving rise to many side effects besides the increased incidence of relapse on these drugs. Therefore, Ag-chitosan-ascorbic acid NCs can be used alone or in conjunction with other medications as a preventive and/or a curative agent against gastric injury.

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CONFLICT OF INTEREST STATEMENT

None of the authors have any known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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