

# The Protective Effect of Curcumin against Nitrosamine-Induced Gastric Oxidative Stress in Rats

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**ABSTRACT:** Curcumin has a wide spectrum of biological, pharmaceutical, and antioxidant effects in cancer experimental models. Nitrosamine is commonly used as an experimental oxidizing agent which induces gastric oxidative stress and gastric carcinogenesis in rats. We examined the antioxidant potential effect of curcumin against nitrosamine-induced gastric oxidative stress in rats. Forty Sprague-Dawley rats were randomly divided into 4 groups (10 rats/group). The control group was fed a standard diet and received a single dose of normal saline, the nitrosamine-treated group was fed a standard diet and received an intraperitoneal injection of nitrosamine at a single dose of 100 mg/kg body weight (b.w.). The other two groups received a daily dose of curcumin (200 mg/kg b.w.) via intra-gastric intubation in the presence or absence of nitrosamine injection. After 16 weeks, all rats were sacrificed, and the gastric tissues were dissected for histopathological examination and for biochemical measurements of oxidative stress indices. Our results showed that nitrosamine causes oxidative stress in gastric tissues as evidenced by glutathione depletion, increased level of lipid peroxides, nitric oxide release, impairment of total antioxidant capacity, DNA oxidative damage, and inhibition of antioxidant enzymes (catalase, glutathione peroxidase, glutathione reductase, and superoxide dismutase). Histopathological findings revealed abnormal gastric architecture in association with nitrosamine injection compared to the non-treated control group. Curcumin significantly suppressed the gastric oxidative damage associated with nitrosamine treatment and mitigated its histopathological effect. These results suggest that curcumin, as an antioxidant, has a therapeutic effect against oxidative stress-mediated gastric diseases.

**Keywords:** curcumin, nitrosamine, oxidative stress, gastric tissue

## INTRODUCTION

Nitrosamines can be formed in food during storage and processing. Nitrosamines are generally formed through chemical reactions of secondary amines such as dimethylamine with nitrate or nitrite (1). *N*-nitrosodimethylamine is one of the most occurring nitrosamines in our diets, and it induces malignant tumors in the esophageal and gastro-intestinal tissues of animal models by causing gene mutation and DNA adductions (2). Nitrates and nitrites are two types of inorganic compounds, which are composed of a single nitrogen atom (N) and several oxygen atoms (O), and the chemical symbols are NO<sub>3</sub> and NO<sub>2</sub> for nitrate and nitrite, respectively (3). It is believed that nitrates are relatively inert until they are reduced to nitrites (4). It was reported that 0.1 mg/d raise of nitrites intake increases the risk of gastric cancer by 7% (5). Re-

cently, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine was reported to induce gastric tumors in animal models (6). It was reported that nitrosamine ingestion leads to gastric oxidative stress by increasing the gastric cellular production of superoxide anion radicals, hydroxyl, and nitrogen dioxide radicals (6). In addition, nitrosamines induce lipid peroxidation in gastric tissues as evidenced by an increase of thiobarbituric acid reactive substances, a commonly used biomarker of oxidative stress (7).

Reactive oxygen species (ROS) are considered a cellular insult by causing irreversible damages to proteins, lipids of cellular organelles, and to DNA which leads to mutations and carcinogenesis (8). Environmental stressors and pathological insults increased the production of ROS and resulted in oxidative stress, a condition in which the levels of ROS are counterbalancing the cellular defense mechanisms (intracellular glutathione and antioxidant

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enzymes) and lead to the pathogenesis of different types of cancers, including gastric cancer (9). Higher intake of dietary antioxidants relative to nitrates is associated with a lower risk of gastric cancer, and the mechanism is attributed antioxidants combating oxidative stress-mediated gastric carcinogenesis (10).

Curcumin is a polyphenolic chemical constituent that is derived from the rhizome of turmeric (*Curcuma longa*) plant, and it has a powerful medicinal application based on its biological effects that range from antioxidant and anti-inflammatory to inhibition of angiogenesis (11). The molecular mechanism of its cellular effects has been shown to have multiple targets and interacting macromolecules within the cell (12). Curcumin has been shown to regulate the production of pro-inflammatory cytokines tumor necrosis factor- $\alpha$  and inhibited the activation of transcription factors nuclear factor- $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1), which regulated the genes for pro-inflammatory mediators and protective antioxidant genes (13). Curcumin inhibited NF- $\kappa$ B activation by blocking phosphorylation of I- $\kappa$ B, through inactivation of I- $\kappa$ B kinase complex (13). Suppression of AP-1 was due to a direct interaction of curcumin with AP-1 binding to its DNA binding motif and due to inhibition of *c-Jun* and *c-fos*, components of AP-1 (14). It is also reported that curcumin suppresses the activity of a number of enzymes such as cytochrome P450 and cyclooxygenase-2 in animal models with no toxicity (15).

Gastric cancer is the leading cause of cancer-related deaths throughout the world (16). Despite advancement in treatment options, the 5-year survival rates for gastric cancer patients is relatively low (17). Gastric cancer risk is thought to arise from gastroesophageal reflux disease, *Helicobacter pylori*-mediated inflammation, or high consumption of nitrosamines containing foods (18). Several preventive therapies are currently used for the treatment of gastric cancer; surgical removal of cancerous lesions, chemoprevention, and anti-inflammatory therapy. However, the role of dietary bioactive agents in the primary prevention of gastric cancer needs further investigation.

Although curcumin has been shown to act as a chemopreventive agent in different types of cancers, including gastric cancer, the mechanism that underlies this protective effect has not been fully elucidated. This study was undertaken to examine the biochemical and cellular role of curcumin as a protective agent against nitrosamine-induced gastric oxidative stress in rats, as an experimental model for chemical-induced gastric oxidative stress.

## MATERIALS AND METHODS

### Drugs and chemicals

Curcumin, nitrosamine, and sodium chloride physiolog-

ical solution were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

### Animals and experimental design

The protocol used in this study was conducted in accordance with international laws and policies and approved by the Animal Ethics Committee at the Sultan Qaboos University (SQU/AEC/2015-16/7). Forty male Sprague-Dawley rats weighing  $200 \pm 5$  g were obtained from the animal breed at the animal house facility, Sultan Qaboos University, Muscat, Oman. The animals were housed in individual cages at standard conditions (temperature  $22 \pm 2^\circ\text{C}$ , relative humidity about 60%, and 12 h light/dark cycles), and all rats were fed a standard diet and given water *ad libitum*. They were acclimatized for a week prior to the experiment and randomly divided into 4 groups ( $n=10$  rats/group). The control group was fed a standard diet and received a single intraperitoneal injection of 0.9% physiological saline; nitrosamine-treated group was fed a standard diet and a single intra-peritoneal dose of nitrosamine [100 mg/kg body weight (b.w.)] dissolved in 0.9% physiological saline. Nitrosamine is commonly used as a chemical agent which induces gastric oxidative stress in rats (6,7). The other two groups were fed a standard diet and received intra-gastric intubation of 1 mL of curcumin mixed with water (200 mg/kg b.w./d) in the presence or absence of nitrosamine injection. Effective doses for curcumin and nitrosamine were selected similar to an earlier work reported in a previous study (19).

### Body weight

Body weight was recorded weekly for the entire duration of the experiment.

### Animals sacrifice

After 16 weeks, the animals were fasted overnight, and animals were anesthetized with a lethal dose of a cocktail containing ketamine (1 mg), xylazine (5 mg), and acepromazine (0.2 mg). The gastric tissues were excised for histopathological examination of any cancer lesions development, and for biochemical measurements of oxidative stress indices.

### Gastric tissue dissection and preparation

The stomach was excised carefully from each rat and was kept on a glass plate in ice jackets, and then divided into two parts symmetrically along the greater and lesser curves. Part one was rinsed with ice-cold physiological saline and was processed as follow: 50 mg was immediately homogenized in 100 mM potassium phosphate buffer (pH 7.4) using a Potter-Elvehjem tissue homogenizer (Thomas Scientific, Swedesboro, NJ, USA). Then 20 mg was used for DNA extraction as described below. The

second part was fixed flat in 10% formalin solution (Thermo Fisher Scientific, Fair Lawn, NJ, USA) overnight and used for histopathological examination and for electron microscopic examination.

### Histopathological examination

The formalin-fixed gastric tissues were dehydrated in increasing concentrations of ethanol, cleared with xylene and embedded in paraffin. Following tissue processing and paraffin embedding, the fixed gastric tissues were sectioned on a microtome to 4  $\mu\text{m}$  thickness and placed on glass slides which were then stained with hematoxylin-eosin and examined for architecture histology under light microscope examination.

### Biochemical measurements of cellular oxidative stress markers

The homogenized gastric tissue was centrifuged at 10,000  $g$  at 4°C for 30 min. The resulting supernatant was separated into aliquots and used for the determination of reduced glutathione (GSH, Catalog #K464), lipid peroxidation (malondialdehyde, Catalog#K739), total antioxidant capacity (TAC, Catalog #K274), and antioxidant enzymes activities [catalase (CAT, Catalog #K773), glutathione reductase (GR, Catalog #K761), glutathione peroxidase (GPx, Catalog #K762), superoxide dismutase (SOD, Catalog #K335)]. All assay kits were conducted based on the manufacture supplier (BioVision Inc., Milpitas, CA, USA).

### Protein analysis

Protein content of gastric tissue homogenates was assayed by the method of Lowry et al. (20) using bovine serum albumin as a standard.

### DNA oxidative damage using 8-hydroxydeoxyguanosine (8-OHdG) assay

Formation of 8-OHdG is a ubiquitous marker of oxidative DNA damage and was measured for DNA samples extracted from all gastric tissues using a commercial DNA Damage enzyme-linked immunosorbent assay kit (OxiSelect™ Oxidative, Catalog# STA-320, Cell Biolabs Inc., San Diego, CA, USA), the quantity of 8-OHdG in the extracted DNA of each gastric tissue (ng/mL) was measured at 450 nm and quantified by comparison with (8-OHdG) standard curve. Extraction of DNA was carried out according to the method of Sureh et al. (21). Briefly, 20 mg of gastric tissue was placed in Eppendorf tubes and lysed with 600  $\mu\text{L}$  buffer [50 mM NaCl, 1 mM Na<sub>2</sub>ethylenediaminetetraacetic acid (EDTA), and 0.5% sodium dodecyl sulfate, pH 8.3] and gently shaken. The mixture was incubated overnight at 37°C, 20  $\mu\text{L}$  of saturated NaCl was added to the sample, and samples were then shaken and centrifuged at 12,000 rpm for 10 min.

The supernatant was transferred to a new Eppendorf tube and DNA was precipitated by 600  $\mu\text{L}$  cold isopropanol. The mixture was inverted several times until fine fibers appeared, and then centrifuged for 5 min at 12,000 rpm. The supernatant was removed, and the pellets were washed with 500  $\mu\text{L}$  of 70% ethyl alcohol then centrifuged at 12,000 rpm for another 5 min. After centrifugation, the alcohol was decanted, and pellets were re-suspended in 50  $\mu\text{L}$  of Tris-EDTA buffer solution (10 mM Tris and 1 mM EDTA, pH 8.0).

### Statistical analysis

The results are expressed as means  $\pm$  standard deviation (SD) and analyzed using GraphPad Prism (version 5.03, GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Turkey's test was used for means comparisons, and a  $P$ -value of less than 0.05 is considered significant.

## RESULTS

The food intake was recorded weekly for the duration of the experiment. The consumption of food in the control rats was  $18 \pm 2$  g/week. The same pattern was observed for the curcumin supplemented group and nitrosamine-injected group indicating that they had no negative interactive effects on food intake. Furthermore, curcumin supplementation to the nitrosamine-injected rats did not cause any change in the food intake as compared to the control group,  $18 \pm 2$  g/week.

Fig. 1 represents weekly body weight changes in the experimental groups throughout the duration of the experiment. There was a significant weight reduction in

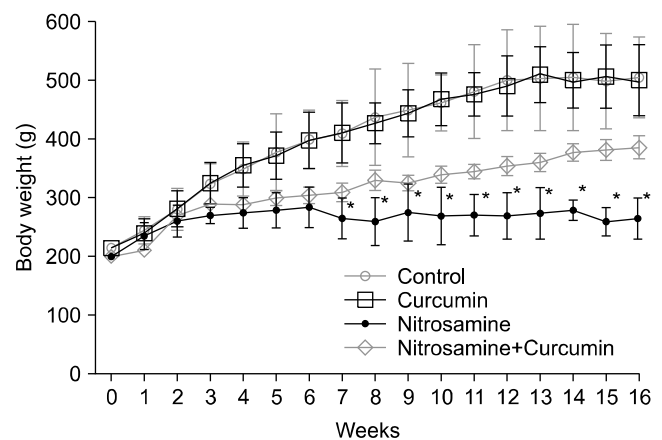
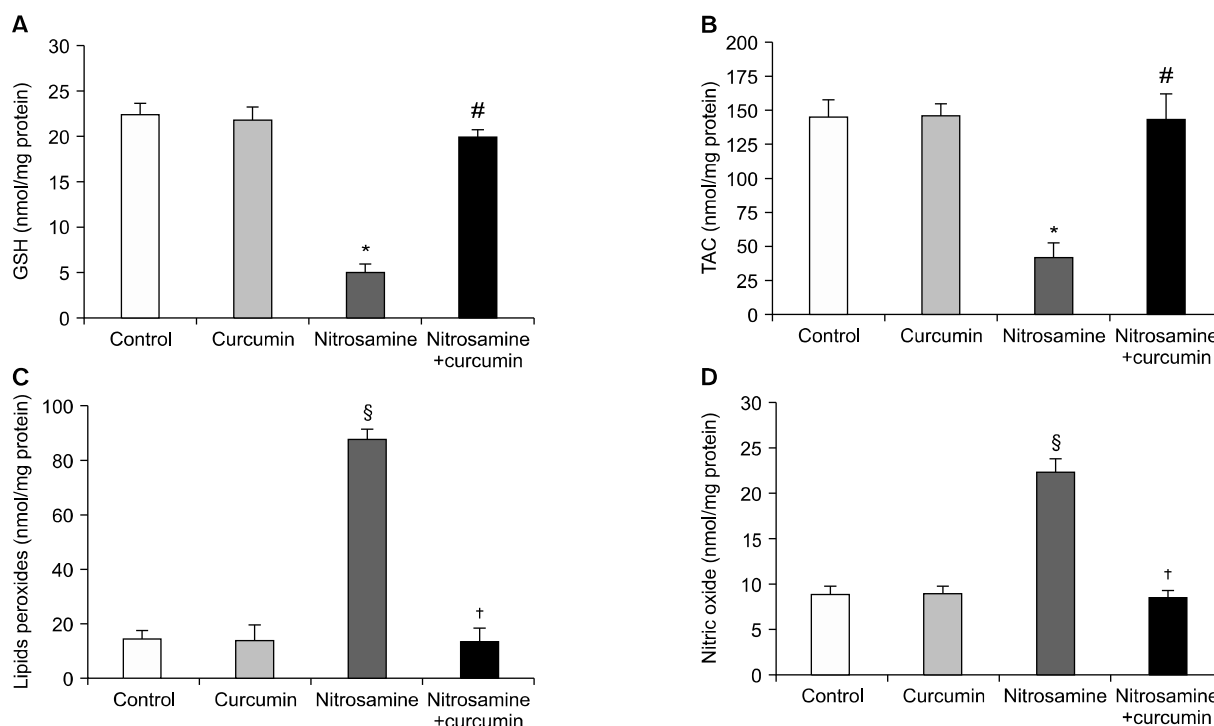


Fig. 1. Changes in body weight of rats supplemented with curcumin in the presence or absence of nitrosamine treatment. Animals in the four groups were examined for the changes in their body weight every week for 16 weeks. Each bar represents the mean  $\pm$  SD of 10 rats/group. \*Significantly lower as compared to control group and curcumin+nitrosamine group,  $P < 0.05$ .



**Fig. 2.** Measurements of cellular oxidative stress indices in gastric tissue homogenates of rats supplemented with curcumin in the presence or absence of nitrosamine carcinogen. (A) glutathione (GSH), (B) total antioxidant capacity (TAC), (C) lipid peroxides, and (D) nitric oxide (NO). Data are expressed as mean $\pm$ SD. Significantly \*lower and §higher as compared to control group, and †lower and #higher than nitrosamine injected group,  $P < 0.05$ .

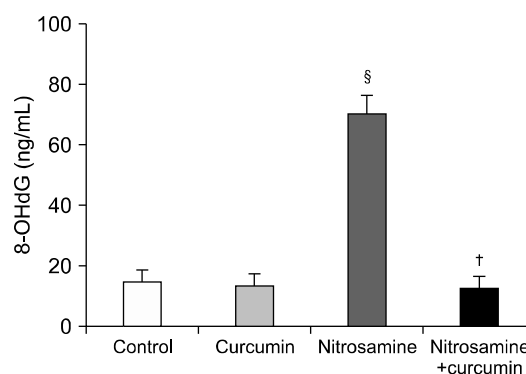
the experimental group that received nitrosamine injection compared to control group,  $P < 0.05$ . This weight reduction might be attributed to the nitrosamine-mediated oxidative damage in gastric tissue which compromised the digestion function ability of the nitrosamine-injected rats and caused their weight loss. Curcumin showed no effect on body weight changes throughout the experiment compared to the control group,  $P > 0.05$ . Meanwhile, curcumin supplementation in the nitrosamine injected group resulted in improvement of the body weight loss as it was detected at 3 weeks and became significantly higher than nitrosamine group at 6 weeks to the end of the experiment,  $P < 0.05$ .

Nitrosamine acted as an oxidizing agent in the nitrosamine-injected group and resulted in significant reduction of the intracellular GSH levels, impairment of TAC, increase in the lipids peroxides, and release of nitric oxide levels compared to the control group,  $P < 0.05$  (Fig. 2). It has been observed that the curcumin supplementation to the nitrosamine-injected group resulted in combating the observed nitrosamine-induced oxidative stress indices specifically by restoring the level of depleted glutathione to a level that is comparable to the control group,  $P > 0.05$  (Fig. 2A). The same trend was observed for the protective effects of curcumin supplementation on abrogating the nitrosamine-mediated effect on TAC, lipids peroxides, and nitric oxide levels (Fig. 2B, 2C, and 2D, respectively).

As illustrated in Fig. 3, nitrosamine caused oxidative

damage to the DNA in the gastric tissues of rats injected with nitrosamine, and the difference was significantly higher than the control group,  $P < 0.05$ . Curcumin supplementation showed a significant reduction in the DNA damage in the nitrosamine-injected group,  $P < 0.05$ .

Curcumin supplementation protected against nitrosamine-induced antioxidant enzymes inhibition in gastric tissue homogenates, Table 1. Nitrosamine caused a significant inhibition in the activities of CAT, GPx, GR, and SOD compared to the control group,  $P < 0.05$ . The treatment of the nitrosamine-injected group with curcumin supplementation had significantly alleviated the nitros-



**Fig. 3.** DNA oxidative damage in gastric tissues of the experimental groups. Data expressed as mean $\pm$ SD. Significantly §higher than control group and †lower than nitrosamine-injected group,  $P < 0.05$ . 8-OHdG, 8-hydroxydeoxyguanosine.

**Table 1.** The effect of curcumin and nitrosamine on antioxidant enzymes in gastric tissue homogenates (unit:  $\mu\text{mol}/\text{min}/\text{mg}$  protein)

Parameter	Control	Nitrosamine	Curcumin	Curcumin+Nitrosamine
CAT	118.42 $\pm$ 6.5	38.69 $\pm$ 4.1*	117.45 $\pm$ 3.9	118.14 $\pm$ 5.6 <sup>#</sup>
GPx	21.38 $\pm$ 1.5	8.91 $\pm$ 0.9*	20.87 $\pm$ 1.4	20.98 $\pm$ 1.1 <sup>#</sup>
GR	11.45 $\pm$ 1.1	4.78 $\pm$ 0.8*	11.22 $\pm$ 0.9	11.38 $\pm$ 0.9 <sup>#</sup>
SOD	84.69 $\pm$ 5.2	28.97 $\pm$ 4.7*	84.15 $\pm$ 4.1	83.94 $\pm$ 3.7 <sup>#</sup>

CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase.

Significantly \*lower than control group and <sup>#</sup>higher than nitrosamine-injected group at  $P < 0.05$ .

amine-associated inhibition of these antioxidant enzymes activities,  $P < 0.05$ , Table 1.

The histopathological sections of the non-treated control group displayed the normal architecture of gastric tissue (Fig. 4A). Nitrosamine injection showed abnormal architecture and arrangement compared to the non-treated control group suggesting pathological changes associated in response to the nitrosamine injection (Fig. 4B). The curcumin supplemented group in the absence of nitrosamine injection showed no pathological changes (Fig. 4C), but the curcumin supplemented group in the presence of nitrosamine showed a dramatic improvement in the histologic appearance similar to the non-treated control rats with no evidence of pathological changes (Fig. 4D).

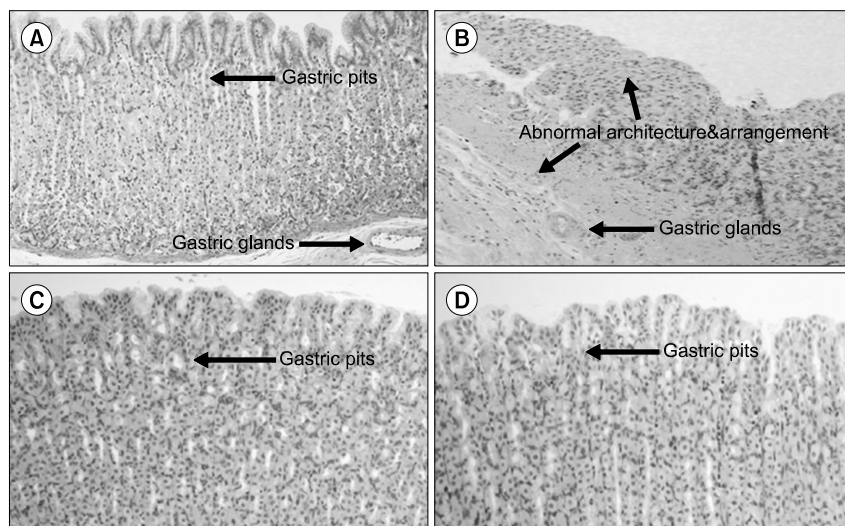
## DISCUSSION

The potential associations between dietary consumption of nitrates, nitrites, nitrosamines, and gastric cancer risk have been investigated by several studies but yielded inconclusive results. The present study elucidated the role of curcumin in alleviating nitrosamine-induced oxidative stress in the examined animal model. It is known that

ROSs are the mediators of oxidative stress and cellular insults by inducing lipid peroxidation in the cells membrane moiety, protein damage, DNA fragmentation, gene mutations, and inhibition of antioxidant enzymes (22). We reported that rats injected with nitrosamine developed oxidative stress in the gastric tissues as evidenced by glutathione depletion, increased level of lipids peroxides, nitric oxide release, impairment of total antioxidant capacity, DNA oxidative damage, and inhibition of antioxidant enzymes (CAT, GPx, GR, and SOD), as well as histopathological changes in the examined gastric tissues.

However, curcumin supplementation had suppressed the oxidative damage associated with nitrosamine injection and mitigated its oxidative stress insult. These findings suggest that curcumin had a gastro-protective effect against nitrosamine-induced oxidative stress and its associated pathogenesis. Our findings are consistent with the well documented role of natural plants in the treatment and prevention of chronic non-communicable diseases, including cancer (23). Curcumin is found ubiquitously in plants and its regular consumption has wide medicinal applications (24). Previous studies have long identified that curcumin prevents generation of cellular oxidative stress by acting as upstream therapeutic barrier to oxidative stress and inflammation, hence offering a novel approach to prevent oxidative stress-induced pathogenesis (25). It is well documented that curcumin scavenges the carcinogenic-induced oxidative stress in different experimental models (26-28), and this is consistent with our reported results which addresses the primary prevention of oxidative stress as a well-known etiological factor for various cancers, including gastric cancer. However, it is essential to conduct human-based clinical trials to evaluate the specific chemopreventive effect of curcumin supplementation.

In conclusion, nitrosamines administration resulted in oxidative stress and abnormal histological morphology



**Fig. 4.** Representative section of gastric tissue architecture histology after haematoxylin and eosin staining (magnification  $\times 40$ ). (A) Control non-treated group with normal gastric architecture. (B) Nitrosamine-injected group showed abnormal architecture and arrangement as compared to the control non-treated group which may suggest oxidative damage in response to nitrosamine injection. (C) Curcumin supplemented group in the absence of nitrosamine injection with no pathological changes. (D) Curcumin supplemented group in the presence of nitrosamine showed dramatic improvement in the histologic appearance similar to the control non-treated rats.

of the examined gastric tissues. Curcumin supplementation significantly mitigated the observed gastric oxidative stress due to nitrosamine injection, and it was found to be significant in regaining all the examined markers to normal as the control non-treated group, indicating a gastro-protective effect. Our study supports the notion that curcumin supplementation has a promising therapeutic effect in the primary prevention of oxidative stress-mediated pathogenesis of gastric diseases, including gastric cancer.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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