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Gastroprotective and antioxidant effects of stachydrine against indomethacin-induced gastric injury via ERK, AKT and iNOS signaling pathways

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Abstract

Stachydrine, a major bioactive alkaloid extracted from *Leonurus heterophyllus*, is a key component of traditional herbal medicine, recognized for its anti-inflammatory and antioxidant properties. In this study, we investigated the gastroprotective effects of stachydrine and its underlying mechanisms in a mouse model of indomethacin (IND)-induced gastric injury. Mice were intragastrically administered IND at a dose of 40 mg/kg, followed 30 minutes later by treatment with varying doses of stachydrine (0, 5, and 10 mg/kg). Six hours post-IND administration, animals were sacrificed for further analysis.

The results demonstrated that stachydrine treatment effectively attenuated IND-induced acute gastric injury, as evidenced by reduced gastric myeloperoxidase activity, and pro-inflammatory cytokine production (TNF-α, IL-6, and IL-1β). Stachydrine also significantly decreased gastric malondialdehyde activity while enhancing superoxide dismutase activity. Furthermore, it suppressed the expression of extracellular signal-regulated kinase (ERK), protein kinase B (AKT), and inducible nitric oxide synthase (iNOS) expressions. These findings indicate that stachydrine confers gastroprotection against IND-induced gastric injury, potentially by suppressing inflammatory and oxidative stress responses, inhibiting the ERK, AKT and iNOS signaling pathways. Thus, stachydrine may serve as a promising candidate for the treatment of IND-induced gastric injury.

Keywords: stachydrine; indomethacin; gastric injury; inflammation; ERK; AKT; iNOS

Introduction

Gastric ulcer is a prevalent condition in the global population. It is marked by damage to the gastric mucosa, leading to perforations and bleeding [1]. Various factors, both endogenous and exogenous, such as acid, pepsin, stress, alcohol, non-steroidal anti-inflammatory drugs (NSAIDs), Helicobacter pylori infection, and smoking, contribute to the development and exacerbation of gastric ulcers [2].

NSAIDs possess analgesic and antipyretic properties and are a significant contributor to gastric ulcer disease. Indomethacin (IND), a type of NSAIDs, is widely prescribed for the management of pain and inflammation such as gout, arthritis, and tendonitis. However, its use can damage the gastric mucosa primarily by reducing prostaglandin synthesis through the inhibition of cyclooxygenase (COX) enzymes, with additional effects mediated by COX-independent pathways, decreasing local blood flow, and inhibiting tissue regeneration [3]. Additionally, cytotoxicity of NSAIDs contributes to gastric injury by elevating intracellular reactive oxygen species (ROS) levels and promoting the release of inflammatory cytokines [4]. The pathogenesis of gastric ulcerative lesions is multifactorial and remains incompletely understood. Although various synthetic anti-ulcer drugs are available, their use is frequently accompanied by adverse effects ranging from mild to severe [5].

Conventional treatments for NSAID-induced gastric injury, such as proton pump inhibitors (PPIs), have been linked to adverse effects such as fractures [6], stroke [7], and an increased risk of gastric cancer [8]. Studies suggest that PPIs may worsen NSAID-induced intestinal injury by altering intestinal microflora [9]. These concerns highlight the necessity of identifying safe and effective natural therapies to address NSAID-related gastrointestinal complications.

Leonurus heterophyllus, commonly known as motherwort, is a traditional herbal medicine primarily used for gynecological disorders [10]. Stachydrine (ST), a bioactive constituent of L. heterophyllus, exhibits a broad spectrum of pharmacological activities, including antioxidant, anti-inflammatory, anticancer, and cardioprotective effects [11-13]. Research has shown that ST significantly suppressed IL-1β-induced inflammation with decreased levels of inflammatory mediators, such as inducible nitric oxide synthase (iNOS) and COX-2 in osteoarthritis chondrocytes [14]. These findings highlight the potential role of ST in modulating inflammatory responses and oxidative stress. However, its therapeutic effects on IND-induced gastric injury have not been investigated. Therefore, this study aimed to elucidate the underlying mechanisms of ST in a mouse model of IND-induced gastric injury.

Materials and methods

Animals

Adult male C57BL/6 mice were obtained from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). Animals were maintained under standardized housing conditions with a 12-hour light/dark cycle and had free access to laboratory chow and water. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial

Hospital (Approval No. 2024120402). All procedures adhered to the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was conducted in accordance with the ARRIVE 2.0 guidelines.

Experimental design

Mice were randomly divided into six groups (n = 6 each). Among them, four groups received IND (40 mg/kg) via intragastric administration; thirty minutes later, these animals were injected intraperitoneally with ST at doses of 0, 5, 10 mg/kg, or with lansoprazole (LPZ, 30 mg/kg). ST used in this study was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) and dissolved in sterile phosphate-buffered saline (PBS) immediately before administration to achieve a concentration of 1 mg/mL. The two control groups that remained received an equal volume of normal saline, followed by either PBS or ST (10 mg/kg). After six hours of treatment, all animals were euthanized by cervical dislocation under 4–5% isoflurane anesthesia, and body weights at the time of sacrifice ranged from 25 to 30 g. Then both blood and gastric tissues were collected for subsequent analyses. In this experimental setting, a single dose administration with a 6-hour evaluation interval was selected according to the rationale described previously [15].

Macroscopic manifestations of stomach damage

Stomachs were excised from the mice, sectioned along the greater curvature and washed thoroughly with cold saline. Residual fluid was removed with gauze, after which the tissues were placed on a clean white background for optimal visualization and subsequently photographed.

Histopathological evaluation of gastric injury

Stomach tissues were immersed in 10% neutral-buffered formalin for 48

hours, followed by dehydration through a graded ethanol series, and subsequently embedded in paraffin. Tissue slices measuring 4 μm in thickness were prepared and stained with hematoxylin and eosin (H&E). Histopathological alterations associated with gastric injury were assessed using a light microscope.

Immunohistochemical analysis on gastric tissues

Gastric sections embedded in paraffin were deparaffinized, blocked for 30 minutes, and incubated at 37 °C for 2 hours with primary antibodies targeting NF-κB, iNOS, and COX-2 (BD Biosciences Pharmingen, San Diego, CA, USA). Following a 5-minute wash with PBS, the sections were incubated for 1 hour with secondary antibodies conjugated to biotin- and streptavidin-horseradish peroxidase. Color development was achieved using a DAB substrate, and nuclei were counterstained with hematoxylin following the manufacturer's protocol (Epredia™ DAB Quanto Detection System, Kalamazoo, MI, USA). All samples were processed under identical conditions and examined with a light microscope.

For quantification, digital images were captured under identical exposure settings. IHC staining intensity was quantified using ImageJ software (NIH, Bethesda, MD, USA). The color deconvolution plugin was applied to distinguish NF-κB staining from hematoxylin background and separate DAB staining. A threshold was set to identify positively stained regions, and the mean percentage of DAB-positive area within each field was calculated. At least five non-overlapping fields per sample were analyzed, and the average value was used for statistical comparison across groups.

Measurement of tissue myeloperoxidase (MPO) activity

MPO levels, reflecting neutrophil-mediated oxidative stress, were

determined in gastric samples. Tissues were homogenized and centrifuged at 15,000 × g for 15 minutes at 4 °C, after which the pellet was resuspended in 50 mM potassium phosphate buffer (pH 6.0). Following incubation at 60 °C for 2 hours, sonication, and three freeze–thaw cycles, a second centrifugation was conducted. The supernatant was mixed with o-dianisidine dihydrochloride, hydrogen peroxide, and phosphate buffer, and absorbance at 460 nm was recorded over 5 minutes.

Measurement of TNF-α, IL-6, and IL-1β levels in gastric tissues

TNF-α, IL-6, and IL-1β concentrations in gastric homogenates were measured using ELISA kits following the manufacturer's protocols (R&D Systems, McKinley, Minneapolis, USA). Absorbance was read at 450 nm, with signal intensity corresponding to cytokine concentration.

Measurement of oxidative stress markers in gastric tissues

Gastric samples were homogenized in 10% trichloroacetic acid and centrifuged at 1,000 × g for 15 minutes at 4 °C. The supernatant further clarified by centrifugation at 35,000 × g for 8 minutes. The final extract was analyzed for malondialdehyde (MDA) using a commercial assay kit (Abcam, ab118970, Cambridge, UK) and for superoxide dismutase (SOD) activity with a SOD kit (#706002; Cayman Chemical, Ann Arbor, MI, USA).

Western blot analysis

Homogenized gastric tissues were centrifuged at 13,000 × g for 10 minutes at 4 °C. Protein levels in the supernatant were determined with the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were denatured, separated using SDS-PAGE, and transferred onto PVDF membranes. The membranes were blocked with TBS for 2 hours and incubated overnight at 4 °C with primary antibodies against ERK, p-ERK, AKT, p-AKT, COX-2, and

iNOS (Cell Signaling Technology, MA, USA), washed, and probed with secondary antibodies for 1 hour. Protein signals were detected via enhanced chemiluminescence. (Amersham, Piscataway, NJ, USA)

Statistical analysis

Statistical analyses were performed using GraphPad Prism (v6.0; GraphPad Software Inc., San Diego, CA, USA). Data are presented as the mean ± standard error of the mean (SEM). Group differences were evaluated by one-way ANOVA followed by Tukey–Kramer multiple comparison tests. A *p*-value less than 0.05 was considered statistically significant.

Results

Protective effects of ST against IND-induced gastric damage

To preliminarily assess the protective effect of ST on IND-induced gastric injury, macroscopic and microscopic pathological evaluations were conducted (Fig. 1 and 2). The gastric tissues of the control group displayed normal gross morphology (Fig. 1A and B). In contrast, animals administered IND exhibited prominent mucosal folds with severe erosion, along with evident ulceration and hemorrhagic foci on the gastric mucosa (Fig. 1C). In the group treated with ST (5 and 10 mg/kg), macroscopic examination revealed mild serosal edema, with only slight erosion and scattered bleeding foci on the mucosal surface (Fig. 1D and E). In the group treated with LPZ (30 mg/kg), gastric tissue recovery was comparable to that of the normal control group (Fig. 1F). Histopathological examination with HE staining revealed that the IND group had marked mucosal erosion extending to the lamina muscularis, accompanied by submucosal edema and hemorrhagic infiltration. (Fig. 2A). Treatment with ST (10 mg/kg) demonstrated a notable protective effect, as evidenced by only mild

mucosal erosion, limited epithelial degeneration and necrosis, and slight submucosal edema. In the LPZ group, tissue structure was more intact, with fewer mucosal erosion and submucosal edema. These findings support the gastroprotective properties of ST, as confirmed by histological analysis. The extent of gastric ulcerated area also confirmed these findings (Fig. 2B). The percentage of gastric tissue with ulcerated damage was markedly increased in IND-only group. After ST treatment, the mice showed similar trend and decreased gastric ulcerated areas compared with the IND-only mice.

ST decreases MPO activity in IND-induced gastric damage

To investigate the effects of ST on the inflammatory damage in gastric tissue, we used the MPO expression as a biomarker of neutrophil accumulation. Gastric MPO expression was significantly increased at 6 hours following IND administration compared to that of the control group ($p < 0.005$, Fig. 3), indicating IND-induced neutrophil infiltration. Higher dose of ST treatment (10 mg/kg), administered 30 minutes post-IND, significantly reduced gastric MPO activity relative to the IND-only group ($p < 0.05$).

Effects of ST on gastric TNF-α, IL-6, and IL-1β levels

To assess the expression of pro-inflammatory cytokines following IND-induced gastric injury, results of TNF-α, IL-6, and IL-1β levels are shown in Fig. 4. These three cytokines were significantly elevated at 6 hours post-IND administration compared to controls. Treatment with ST (5 mg/kg) 30 minutes after IND administration didn’t significantly reduced TNF-α, IL-6, and IL-1β levels. However, higher doses of ST (10 mg/kg) and LPZ treatment significantly attenuated these three cytokines.

Effects of ST on oxidative stress markers

IND administration triggered oxidative stress injury and activated antioxidant

defense system. We assessed MDA and SOD levels as markers of oxidative stress in gastric tissues. The MDA concentration was significantly increased in the IND group compared to the normal group ($p < 0.005$) (Fig. 5A). Following ST treatment (5 and 10 mg/kg), MDA levels were markedly reduced relative to those in IND-treated mice ($p < 0.05$). Additionally, excessive oxidative stress suppressed the activity of SOD, a key antioxidant enzyme, with SOD levels notably lower in the IND group than in the normal group ($p < 0.05$) (Fig. 5B). Treatment with higher dose of ST (10 mg/kg) effectively restored gastric SOD activity ($p < 0.05$).

Effect of ST on ERK and AKT expressions in gastric tissues

We evaluated gastric ERK and AKT expressions and its phosphorylation following IND-induced gastric injury. Western blot analysis revealed a significant increase in phospho-ERK and -AKT levels after a single IND exposure compared to controls ($p < 0.005$; Fig. 6A and B). Administration of 2 different doses of ST (5 and 10 mg/kg) 30 minutes post-IND showed significantly decreased phospho-ERK and -AKT levels compared to IND treatment alone.

Quantification of NF-κB immunostaining in gastric tissue

Immunohistochemical staining revealed minimal NF-κB immunoreactivity in gastric tissues from the vehicle and ST (10 mg/kg) groups (Fig. 7A). In contrast, IND administration markedly increased NF-κB staining, predominantly localized within the gastric mucosa. Treatment with ST significantly reduced NF-κB immunoreactivity in IND-challenged mice, with a more pronounced reduction observed at the higher dose. Lansoprazole treatment also attenuated NF-κB expression compared with the IND group. ImageJ-based quantification of DAB staining revealed that expression of NF-κB was markedly increased in

the IND group compared to the normal group ($p < 0.005$) (Fig. 7B). Following ST treatment (5 and 10 mg/kg), its immunoreactivity was substantially decreased in the ST-treated groups relative to the IND group ($p < 0.005$). Measurement of the stained area fraction demonstrated a reduction in NF-κB activation, consistent with the histopathological observations.

Effect of ST on iNOS and COX-2 expressions in gastric tissues

We also evaluated the expression of gastric iNOS and COX-2 following IND-induced gastric injury. We conducted western blot analyses and immunohistochemical staining utilizing iNOS and COX-2 antibodies. Western blot analysis revealed significant increases in iNOS and COX-2 levels after a single IND exposure compared to the control group ($p < 0.01$ and $p < 0.005$, respectively) (Figure 8A, B and C). Administration of a low dose of ST (5 mg/kg) 30 minutes post-IND had no notable effect on iNOS and COX-2 levels relative to IND alone. In contrast, a higher ST dose (10 mg/kg) significantly suppressed the IND-induced the expressions of both iNOS and COX-2 ($p < 0.005$ and $p < 0.01$, respectively). In addition, the IND-only group showed increased iNOS and COX-2 expressions in gastric tissues compared to those in the control group (Figure 8D and E). The groups treated with higher dose of ST (10 mg/kg) and LPZ exhibited notably reduced iNOS and COX-2 expressions in the gastric mucosa.

Discussion

With growing recognition of the limitations in conventional therapies for NSAID-induced gastric injury, there is increasing interest in identifying novel preventive or therapeutic strategies derived from natural compounds. This study aimed to investigate its efficacy and underlying mechanisms in a model

of IND-induced gastric injury. Comprehensive analysis revealed that administration of ST, a major bioactive alkaloid purified from *Leonurus heterophyllus*, significantly alleviated ulcer bleeding and mucosal edema, suppressed inflammatory infiltration, lowered pro-inflammatory cytokine levels, and decreased the level of oxidative markers and inflammatory factors. This study also demonstrated that ST effectively reduced IND-induced gastric ulcers, with efficacy comparable to the clinically established anti-ulcer agent LPZ.

IND-induced gastric injury is a widely established preclinical model for evaluating compounds with potential gastroprotective properties. Administration of IND at 40 mg/kg effectively replicated key NSAID-associated gastric lesions, including dyspepsia, ulceration, hemorrhage, and edema [16]. Our study is consistent with those reported in previous studies [17]. These pathological features were prominent in the IND group but markedly attenuated in mice treated with ST or LPZ.

In the present study, IND administration elicited a pronounced inflammatory response in the gastric mucosa, characterized by elevated levels of pro-inflammatory cytokines, increased MPO activity, and notable histopathological alterations observed in H&E-stained sections. These findings align with previous experimental reports [18,19]. TNF-α, a key proinflammatory cytokine, has been previously reported to be markedly upregulated in indomethacin-induced gastric injury [20]. It promotes inflammation by increasing adhesion molecule expression, leading to leukocyte recruitment at the injury site. The resulting neutrophil accumulation produces more ROS, which further damages the gastric mucosa [21]. Our findings indicate that ST treatment attenuated the mucosal inflammatory response in a dose-dependent manner, as evidenced by reduced levels of gastric cytokines and MPO, along with

restoration of normal histological architecture. This protective effect is consistent with documented anti-inflammatory properties of ST. Previous studies have shown that ST provides protective effects against CCl_4-induced liver fibrosis by modulating potent anti-inflammatory activity, as evidenced by the significant downregulation of pro-inflammatory mediators, including IL-6, IL-8, IL-1β, TNF-α, iNOS, and COX-2 [22]. Another study also showed that ST mitigate cardiac hypertrophy and fibrosis in rats by suppressing the production of pro-inflammatory cytokines and NF-κB signaling pathway [13]. Collectively, these findings support the anti-inflammatory and mucosal protective potential of ST in IND-induced gastric injury.

In addition, numerous studies have demonstrated that gastric injury is associated with increased ROS production, impaired cellular proliferation, and heightened inflammatory responses [23,24]. Therefore, regulating ROS levels and modulating inflammation are critical strategies for preventing gastric ulceration. NSAIDs are known to cause direct injury to the gastrointestinal mucosa, primarily through the induction of excessive ROS within the gastrointestinal tract. This oxidative stress disrupts cellular metabolism and leads to tissue damage [4]. MDA, a key byproduct of lipid peroxidation, serves as a reliable biomarker for such oxidative injury. Endogenous antioxidant defenses, such as SOD, play a critical role in neutralizing ROS and maintaining redox balance [25]. In the present study, IND administration significantly elevated ROS and MDA levels while suppressing SOD activity, consistent with previously reported findings on IND-induced gastric injury [26,27]. Notably, treatment with ST ameliorated these effects, indicating its potent antioxidant capacity in mitigating IND-induced gastric mucosal damage in mice.

Nitric oxide (NO) is a well-characterized pro-inflammatory signaling

molecule produced during inflammatory responses. iNOS, one of three isoforms of NOS, is activated in response to inflammatory cytokines and other stimuli, such as indomethacin or alcohol. Once induced, iNOS continuously produces NO until the enzyme is degraded [28]. As previously reported, the expression of iNOS is significantly elevated in indomethacin-induced gastric injury [29]. In the present study, western blot analysis revealed a significant increase of iNOS in the IND group in gastric tissues. Treatment with ST markedly reduced expression, indicating its inhibitory effect on this pro-inflammatory mediator. This finding is consistent with prior in vitro studies demonstrating that ST suppresses iNOS expression and reduces inflammatory response in osteoarthritis chondrocytes [30].

Oxidative stress plays a critical role in activating mitogen-activated protein kinase (MAPK) pathways. including the MAPK and NF-kB pathways. These intracellular signaling cascades, in turn, regulate the cellular response to oxidative stress and modulate the production of various inflammatory mediators. Previous studies have demonstrated that IND-induced gastric injury is associated with the activation of MAPK/NF-kB, and targeting MAPK signaling is considered a promising therapeutic strategy for gastric injury [31]. ERK is one important member of the MAPK family that is involved in oxidative stress formation and modulating gastric mucosal injury and repair [32]. TNF-α and IL-6 serve both as upstream activators and downstream effectors of the MAPK signaling cascade. Their overexpression further promotes the phosphorylation of ERK and NF-kB activation, contributing to the progression of tissue damage [33]. In addition, AKT is a key intracellular signaling axis that influences a broad range of biological functions, including regulation of inflammation and promotion of cell survival during tissue injury [34]. Evidence

from previous studies indicates that activation of the AKT pathway can suppress pro-inflammatory signaling and improve outcomes in models of sepsis as well as ischemia–reperfusion injury [35]. Another study reported that ST mitigated neuronal damage after traumatic brain injury in rats, an effect associated with suppression of PI3K/AKT pathway activity [36]. A recent study also reported that ST can induce apoptosis and inhibit ERK and AKT signaling in various cell types [37]. In the present study, we investigated the effect of ST on MAPK activation, focusing on ERK, and AKT pathways. Western blot analysis of animal models revealed a significant increase in phosphorylated ERK expression following IND exposure. Notably, low and high doses of ST markedly reduced ERK and AKT phosphorylation, suggesting that its protective effect against IND-induced gastric injury may be mediated through modulation of the ERK and AKT signaling pathways.

Conclusions

This study demonstrated that ST effectively attenuates IND-induced gastric injury in a murine model. The gastroprotective effects of ST are mediated through its anti-inflammatory and antioxidant properties, which involve inhibition of the ERK, AKT, and iNOS signaling pathways. These findings suggest that ST holds promise as a potential therapeutic agent for the clinical management of IND-induced gastric injury.

Author contributions

L.F.C., Y.H.P. and L.C.C. conceived and designed the experiments; L H.C. and L.H.T. performed the experiments; L.F.C and L.H.C. contributed to the statistical data analysis; L.F.C., Y.H.P, L.H.T. and L.C.C. wrote the manuscript;

L.C.C. corrected the manuscript. All authors have read and approved the manuscript for publication.

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Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

Ethical Approval and Consent to participate

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital (Approval number: 2024120402). The experiments were conducted in strict compliance with the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the study was conducted in accordance with the ARRIVE 2.0 guidelines.

Consent for publication

The paper was read and approved by all writers.

Declaration of Competing Interest

All the authors declare no conflict of interest.

Reference

1 Kangwan, N., Park, J. M., Kim, E. H. & Hahm, K. B. Quality of healing of gastric ulcers: Natural products beyond acid suppression. *World J Gastrointest Pathophysiol* **5**, 40-47, doi:10.4291/wjgp.v5.i1.40 (2014).

2 Syam, A. F., Sadikin, M., Wanandi, S. I. & Rani, A. A. Molecular mechanism on healing process of peptic ulcer. *Acta Med Indones* **41**, 95-98 (2009).

3 Sostres, C., Gargallo, C. J. & Lanas, A. Nonsteroidal anti-inflammatory drugs and upper and lower gastrointestinal mucosal damage. *Arthritis Res Ther* **15 Suppl 3**, S3, doi:10.1186/ar4175 (2013).

4 Maziero Alves, G. *et al.* Sildenafil attenuates nonsteroidal anti-inflammatory-induced gastric ulceration in mice via antioxidant and antigenotoxic mechanisms. *Clin Exp Pharmacol Physiol* **48**, 401-411, doi:10.1111/1440-1681.13414 (2021).

5 Koyyada, A. Long-term use of proton pump inhibitors as a risk factor for various adverse manifestations. *Therapie* **76**, 13-21, doi:10.1016/j.therap.2020.06.019 (2021).

6 Yang, Y. X., Lewis, J. D., Epstein, S. & Metz, D. C. Long-term proton pump inhibitor therapy and risk of hip fracture. *JAMA* **296**, 2947-2953, doi:10.1001/jama.296.24.2947 (2006).

7 Sherwood, M. W. *et al.* Individual Proton Pump Inhibitors and Outcomes in Patients With Coronary Artery Disease on Dual Antiplatelet Therapy: A Systematic Review. *J Am Heart Assoc* **4**, doi:10.1161/JAHA.115.002245 (2015).

8 Brusselaers, N., Wahlin, K., Engstrand, L. & Lagergren, J. Maintenance

therapy with proton pump inhibitors and risk of gastric cancer: a nationwide population-based cohort study in Sweden. *BMJ Open* **7**, e017739, doi:10.1136/bmjopen-2017-017739 (2017).

9 Nadatani, Y. *et al.* Gastric acid inhibitor aggravates indomethacin-induced small intestinal injury via reducing Lactobacillus johnsonii. *Sci Rep* **9**, 17490, doi:10.1038/s41598-019-53559-7 (2019).

10 Xia, W. T. *et al.* Motherwort injection in preventing post-abortion hemorrhage after induced abortion: A multi-center, prospective, randomized controlled trial. *Explore (NY)* **16**, 110-115, doi:10.1016/j.explore.2019.08.004 (2020).

11 Hu, Y., Mao, A., Yu, Z. & He, K. Anti-endotoxin and anti-inflammatory effects of Chinese herbal medicinal alkaloid ingredients in vivo. *Microb Pathog* **99**, 51-55, doi:10.1016/j.micpath.2016.08.006 (2016).

12 Cao, T. T. *et al.* Stachydrine Protects Against Pressure Overload-Induced Cardiac Hypertrophy by Suppressing Autophagy. *Cell Physiol Biochem* **42**, 103-114, doi:10.1159/000477119 (2017).

13 Zhao, L. *et al.* Stachydrine ameliorates isoproterenol-induced cardiac hypertrophy and fibrosis by suppressing inflammation and oxidative stress through inhibiting NF-kappaB and JAK/STAT signaling pathways in rats. *Int Immunopharmacol* **48**, 102-109, doi:10.1016/j.intimp.2017.05.002 (2017).

14 Wu, H., Zhang, M., Li, W., Zhu, S. & Zhang, D. Stachydrine attenuates IL-1β-induced inflammatory response in osteoarthritis chondrocytes through the NF-κB signaling pathway. *Chem Biol Interact* **326**, 109136, doi:10.1016/j.cbi.2020.109136 (2020).

15 Yamagishi, Y., Saiki, R., Yoshimi, T., Kudo, T. & Ito, K. Gastroprotective

Effect of Enteral Nutrition Formula in Mice Injected Subcutaneously with Indomethacin. *Nutrients* **13**, doi:10.3390/nu13093297 (2021).

16 Lanas, A., Boers, M. & Nuevo, J. Gastrointestinal events in at-risk patients starting non-steroidal anti-inflammatory drugs (NSAIDs) for rheumatic diseases: the EVIDENCE study of European routine practice. *Ann Rheum Dis* **74**, 675-681, doi:10.1136/annrheumdis-2013-204155 (2015).

17 Ko, I. G. *et al.* Evaluating the mucoprotective effect of polydeoxyribonucleotide against indomethacin-induced gastropathy via the MAPK/NF-kappaB signaling pathway in rats. *Eur J Pharmacol* **874**, 172952, doi:10.1016/j.ejphar.2020.172952 (2020).

18 Ahmed, M. A. E., Mohanad, M., Ahmed, A. A. E., Aboulhoda, B. E. & El-Awdan, S. A. Mechanistic insights into the protective effects of chlorogenic acid against indomethacin-induced gastric ulcer in rats: Modulation of the cross talk between autophagy and apoptosis signaling. *Life Sciences* **275**, 119370, doi:https://doi.org/10.1016/j.lfs.2021.119370 (2021).

19 Wei, Y. *et al.* Zuojin Pill ameliorates inflammation in indomethacin-induced gastric injury via inhibition of MAPK pathway. *J Ethnopharmacol* **275**, 114103, doi:10.1016/j.jep.2021.114103 (2021).

20 Souza, M. H., Lemos, H. P., Oliveira, R. B. & Cunha, F. Q. Gastric damage and granulocyte infiltration induced by indomethacin in tumour necrosis factor receptor 1 (TNF-R1) or inducible nitric oxide synthase (iNOS) deficient mice. *Gut* **53**, 791-796, doi:10.1136/gut.2002.012930 (2004).

21 Musumba, C., Pritchard, D. M. & Pirmohamed, M. Review article: cellular and molecular mechanisms of NSAID-induced peptic ulcers. *Aliment*

Pharmacol Ther **30**, 517-531, doi:10.1111/j.1365-2036.2009.04086.x (2009).

22 Zhang, J. *et al.* Stachydrine ameliorates carbon tetrachloride-induced hepatic fibrosis by inhibiting inflammation, oxidative stress and regulating MMPs/TIMPs system in rats. *Biomed Pharmacother* **97**, 1586-1594, doi:10.1016/j.biopha.2017.11.117 (2018).

23 El-Ashmawy, N. E., Khedr, E. G., El-Bahrawy, H. A. & Selim, H. M. Gastroprotective effect of garlic in indomethacin induced gastric ulcer in rats. *Nutrition* **32**, 849-854, doi:10.1016/j.nut.2016.01.010 (2016).

24 Cicek, B. *et al.* Artichoke (Cynara Scolymus) Methanolic Leaf Extract Alleviates Diethylnitrosamine-Induced Toxicity in BALB/c Mouse Brain: Involvement of Oxidative Stress and Apoptotically Related Klotho/PPARgamma Signaling. *J Pers Med* **12**, doi:10.3390/jpm12122012 (2022).

25 Qiu, L. *et al.* Effect of Cuttlebone on Healing of Indomethacin-Induced Acute Gastric Mucosal Lesions in Rats. *Evid Based Complement Alternat Med* **2020**, 9592608, doi:10.1155/2020/9592608 (2020).

26 Barboza, K. R. M. *et al.* Gastroprotective effect of oral kefir on indomethacin-induced acute gastric lesions in mice: Impact on oxidative stress. *Life Sci* **209**, 370-376, doi:10.1016/j.lfs.2018.08.035 (2018).

27 Ugan, R. A. & Un, H. The Protective Roles of Butein on Indomethacin Induced Gastric Ulcer in Mice. *Eurasian J Med* **52**, 265-270, doi:10.5152/eurasianjmed.2020.20022 (2020).

28 Cinelli, M. A., Do, H. T., Miley, G. P. & Silverman, R. B. Inducible nitric oxide synthase: Regulation, structure, and inhibition. *Med Res Rev* **40**, 158-189, doi:10.1002/med.21599 (2020).

29 Mohamed, Y. T. *et al.* Role of ADMA/DDAH-1 and iNOS/eNOS signaling in the gastroprotective effect of tadalafil against indomethacin-induced gastric injury. *Biomed Pharmacother* **150**, 113026, doi:10.1016/j.biopha.2022.113026 (2022).

30 Wu, H., Zhang, M., Li, W., Zhu, S. & Zhang, D. Stachydrine attenuates IL-1beta-induced inflammatory response in osteoarthritis chondrocytes through the NF-kappaB signaling pathway. *Chem Biol Interact* **326**, 109136, doi:10.1016/j.cbi.2020.109136 (2020).

31 Akanda, M. R. *et al.* Anti-Inflammatory and Gastroprotective Roles of Rabdosia inflexa through Downregulation of Pro-Inflammatory Cytokines and MAPK/NF-kappaB Signaling Pathways. *Int J Mol Sci* **19**, doi:10.3390/ijms19020584 (2018).

32 Wei, Y. *et al.* Dehydroevodiamine ameliorates indomethacin-induced gastric injury via inhibition of ERK and p38 signaling pathway. *Phytomedicine* **93**, 153764, doi:10.1016/j.phymed.2021.153764 (2021).

33 Li, W. *et al.* Anti-inflammatory effect of tetrahydrocoptisine from Corydalis impatiens is a function of possible inhibition of TNF-α, IL-6 and NO production in lipopolysaccharide-stimulated peritoneal macrophages through inhibiting NF-κB activation and MAPK pathway. *European Journal of Pharmacology* **715**, 62-71, doi:https://doi.org/10.1016/j.ejphar.2013.06.017 (2013).

34 Cianciulli, A. *et al.* PI3k/Akt signalling pathway plays a crucial role in the anti-inflammatory effects of curcumin in LPS-activated microglia. *International Immunopharmacology* **36**, doi:10.1016/j.intimp.2016.05.007 (2016).

35 Williams, D. L., Ozment-Skelton, T. & Li, C. Modulation of the

phosphoinositide 3-kinase signaling pathway alters host response to sepsis, inflammation, and ischemia/reperfusion injury. *Shock* **25**, 432-439, doi:10.1097/01.shk.0000209542.76305.55 (2006).

36 Yu, N., Hu, S. & Hao, Z. Benificial Effect of Stachydrine on the Traumatic Brain Injury Induced Neurodegeneration by Attenuating the Expressions of Akt/mTOR/PI3K and TLR4/NFkappa-B Pathway. *Transl Neurosci* **9**, 175-182, doi:10.1515/tnsci-2018-0026 (2018).

37 Wang, M., Shu, Z. J., Wang, Y. & Peng, W. Stachydrine hydrochloride inhibits proliferation and induces apoptosis of breast cancer cells via inhibition of Akt and ERK pathways. *Am J Transl Res* **9**, 1834-1844 (2017).

Figure Legends

Figure 1. Effects of ST treatment on the gross morphology in IND-induced gastric injury.

(A) and (B) Negative control groups showed no lesions of the gastric mucosa; (C) IND induced

ulceration and extensive lesions as elongated bands of hemorrhage; (D) and (E) Treatment with ST 5 and 10 mg/kg revealed mild erosion and scattered bleeding foci on the mucosal surface; (F) Treatment with LPZ 30 mg/kg showed decreased gastric damages in comparison with the ulcer control. ST: stachydrine, IND: indomethacin, LPZ: lansoprazole. Representative images were selected from each group.

Figure 2. Effects of ST treatment on histological analysis in IND-induced gastric injury.

(A) Histopathology of the gastric mucosa of mice on the microscopic appearance. Hematoxylin and

eosin (H&E) staining was conducted on gastric tissues from six groups. Representative images were selected from each group. (B) Bar graph showed quantitative analysis of the percentage of ulcerated area. Each value is mean ± SEM of 6 mice per group. ***$p < 0.005$ vs. control; ###$p < 0.005$ vs. IND alone.

Figure 3. Effects of ST treatment on MPO activity in IND-induced gastric injury.

Mice were administered with IND alone or with different doses of ST (5 and 10 mg/kg) and LPZ 10 mg/kg 30 min after IND injection. Gastric tissues were obtained 6 h after IND injection. MPO activity data are expressed as means ± SEM (n = 6 mice/group). **$p < 0.01$ vs. control; #$p < 0.05$ vs. IND alone.

Figure 4. Effects of ST treatment on gastric cytokine levels of (A) TNF-α, (B) IL-6, (C) IL-1β **in IND-induced gastric injury.**

Results are expressed as mean ± SEM. *$p < 0.05$, ***$p < 0.005$ compared to the control group; #$p < 0.05$, ##$p < 0.01$ compared to the IND group.

Figure 5. Effects of ST treatment on the MDA and SOD levels in the gastric tissue.

Results are expressed as mean ± SEM. *$p < 0.05$, ***$p < 0.005$ compared to the control group; #$p < 0.05$, ###$p < 0.005$ compared to the IND group.

Figure 6. Effects of ST treatment on gastric ERK and AKT expression.

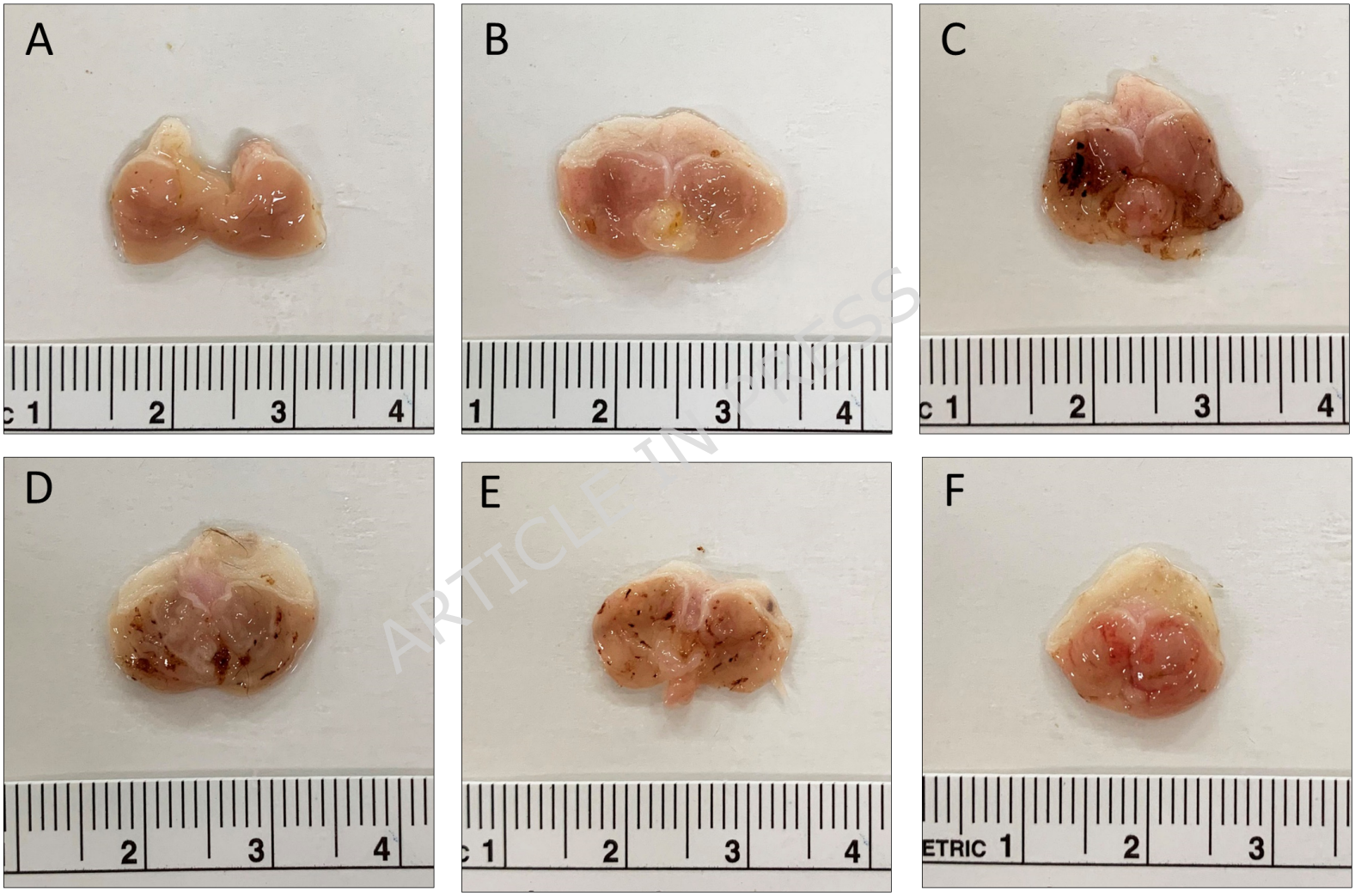
(A) Electrophoretogram of ERK proteins and relative p-ERK protein level in gastric tissue. (B) Electrophoretogram of AKT proteins and relative p-AKT protein level in gastric tissue. Band intensities were assessed via densitometry, and each value is presented as mean ± SEM. ***$p < 0.005$ compared to the control group; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.005$ compared to the IND group.

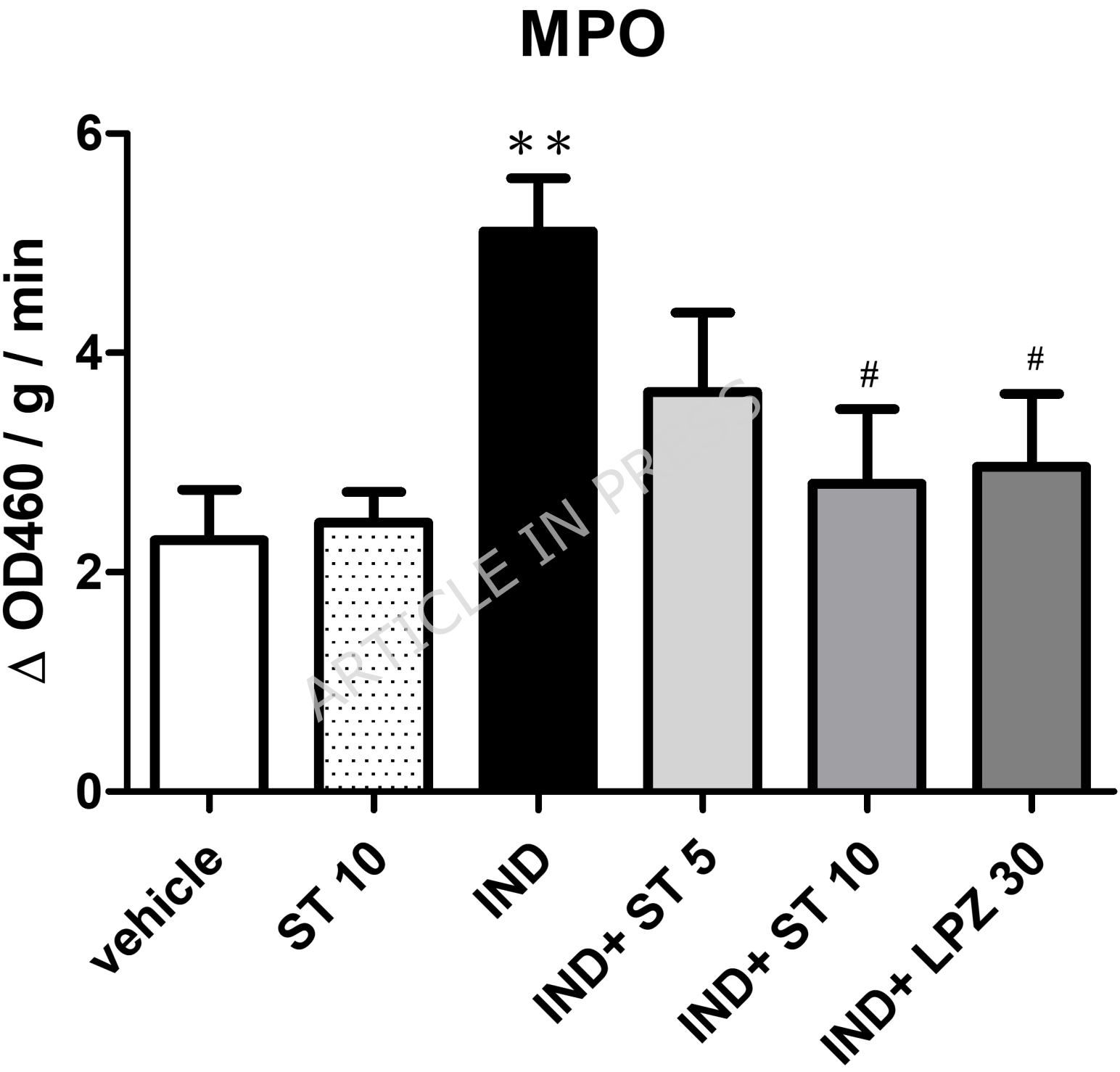
Figure 7. Effects of ST treatment on NF-κB expression in the gastric tissue.

(A) Immunohistochemical staining illustrating gastric NF-κB expressions (brown) across the six groups. Representative images were selected from each group. (B) NF-κB immunostaining was quantified using ImageJ by measuring positive immunostaining area. Bar graph showed quantitative analysis of the percentage of positive area. Each value is mean ± SEM. ***$p < 0.005$ compared to the control group; ###$p < 0.005$ compared to the IND group.

Figure 8. Effects of ST treatment on iNOS and COX-2 expressions in IND-induced gastric injury.

(A) Electrophoretogram of iNOS and COX-2 proteins. (B) Relative iNOS protein level in gastric tissue. (C) Relative COX-2 protein level in gastric tissue. Band intensities were assessed via densitometry, and each value is presented as mean ± SEM. **$p < 0.01$, ***$p < 0.005$ compared to the control group; ##$p < 0.01$, ###$p < 0.005$ compared to the IND group. (D) and (E) Immunohistochemical staining illustrating gastric iNOS and COX-2 expressions (black arrows) across the six groups. Representative images were selected from each group.





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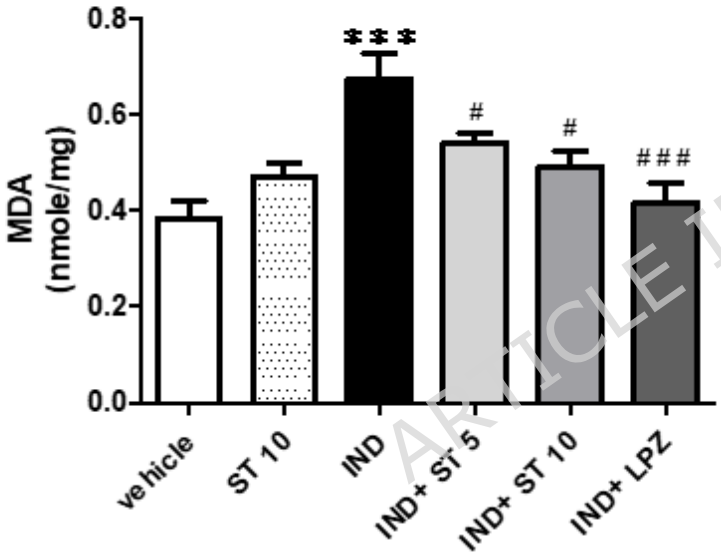

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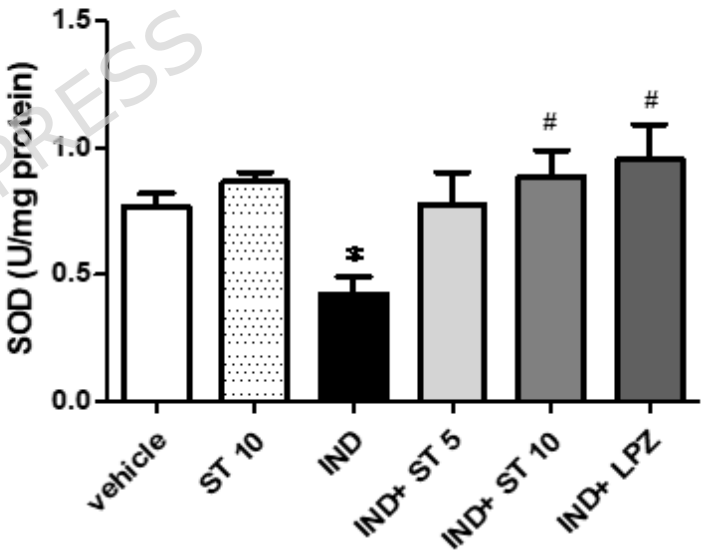

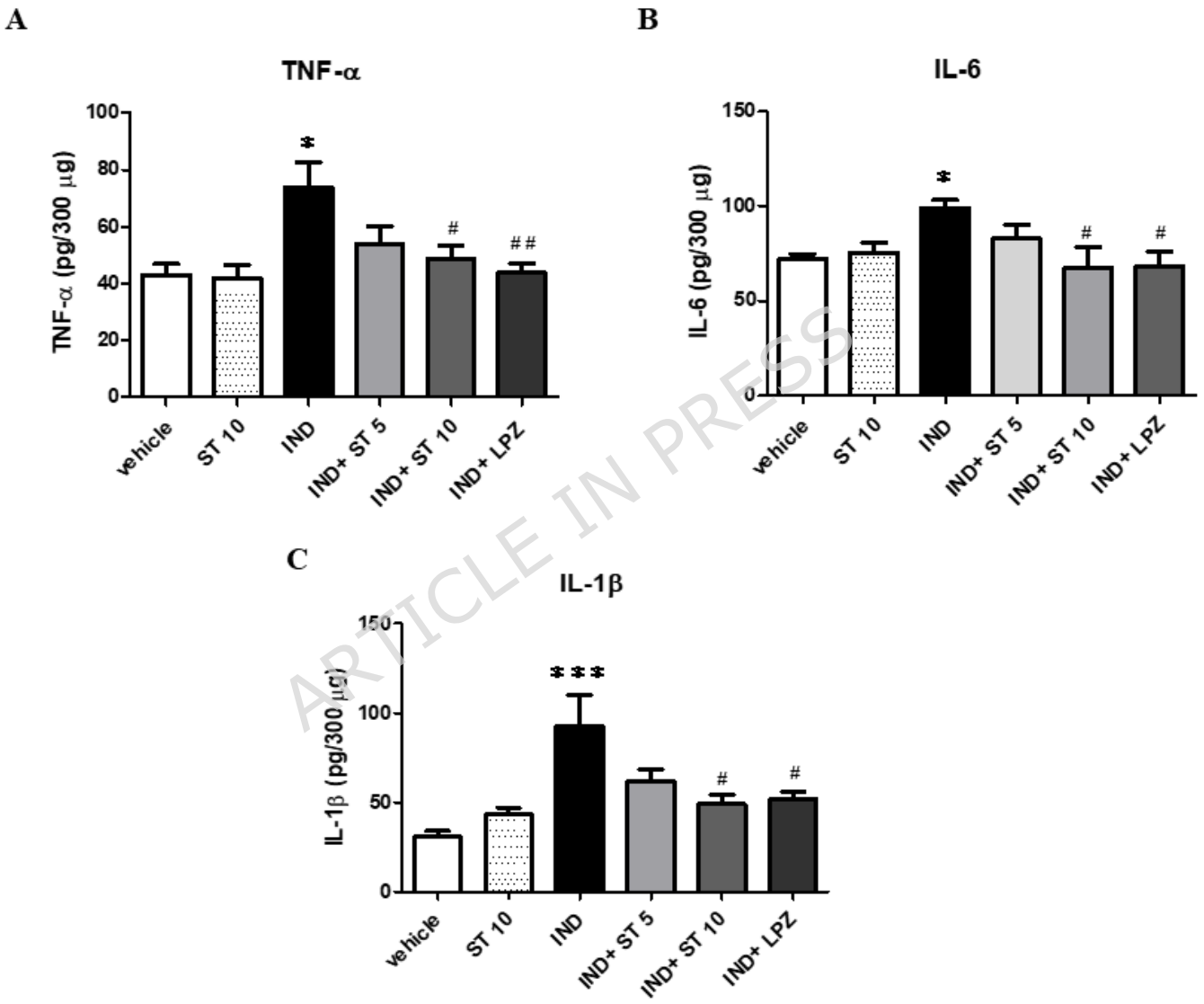

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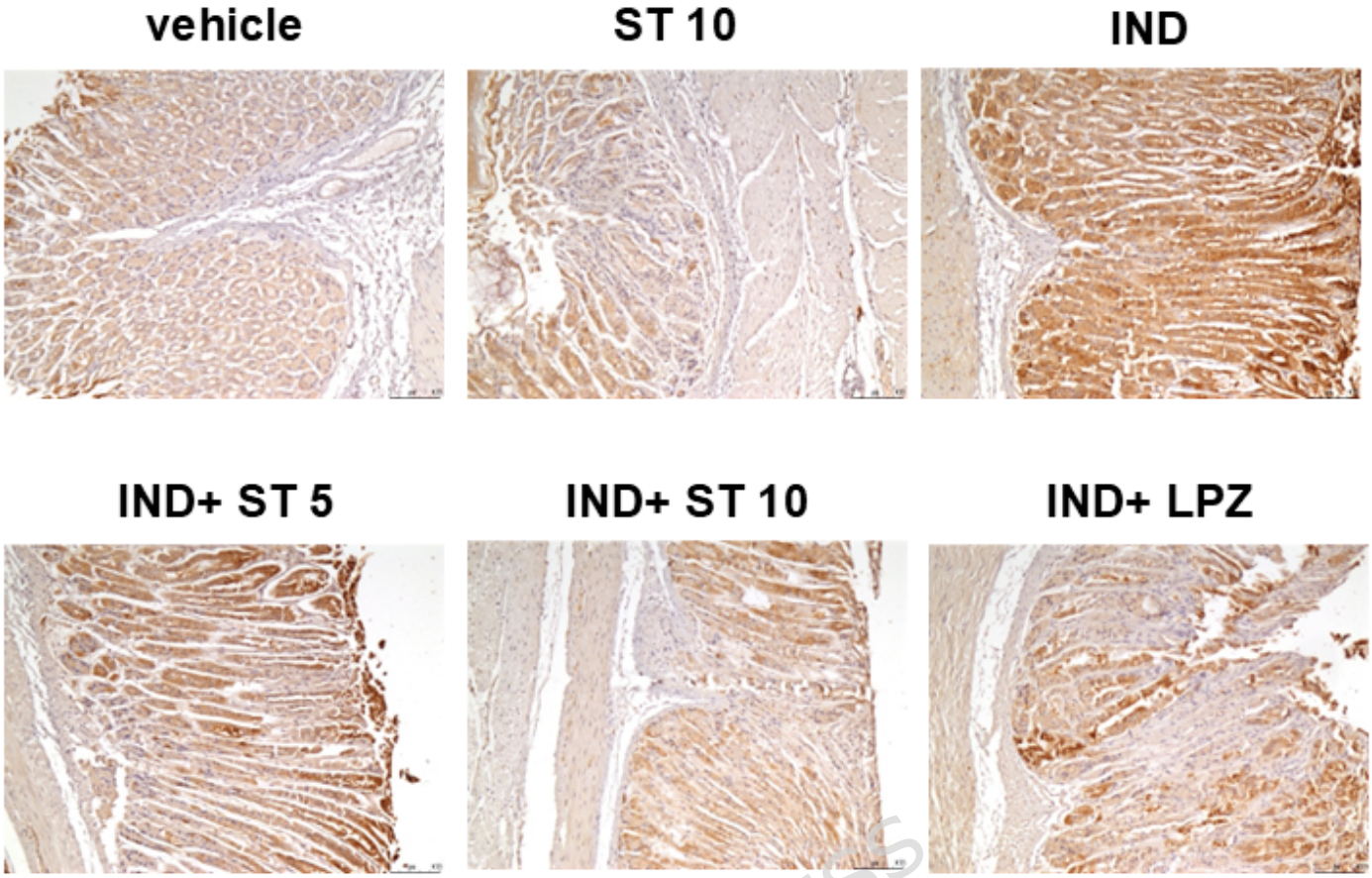

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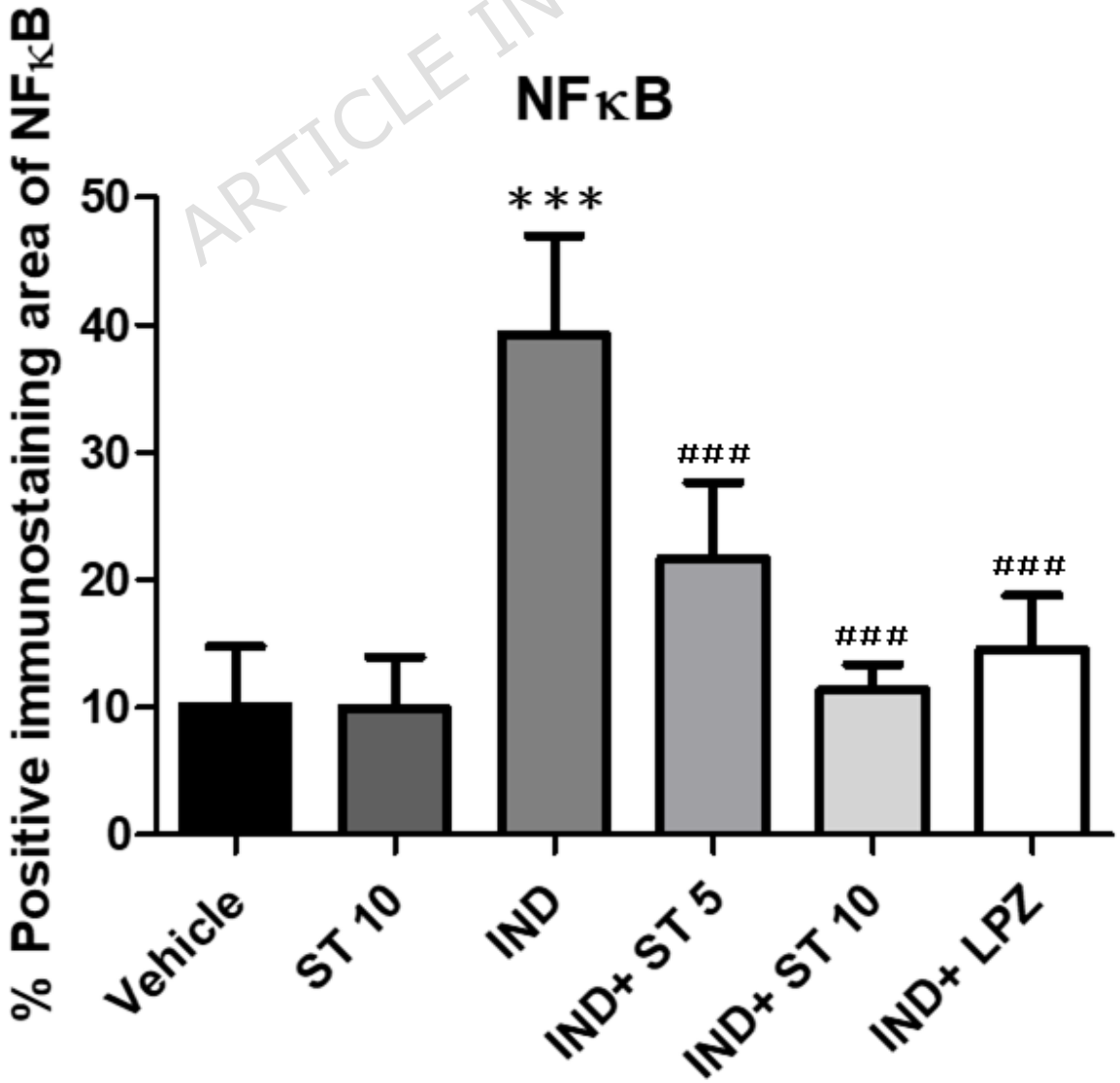

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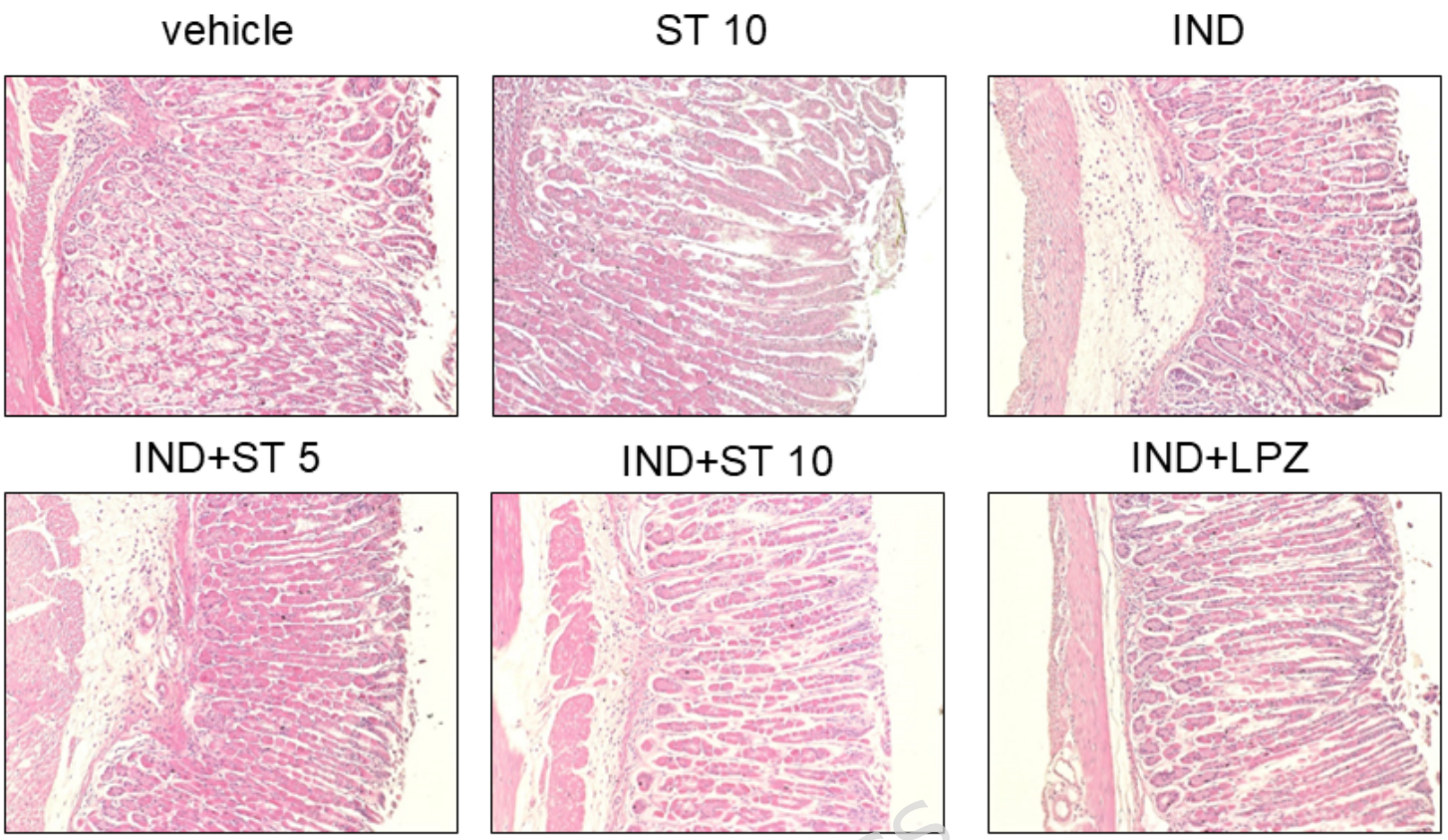

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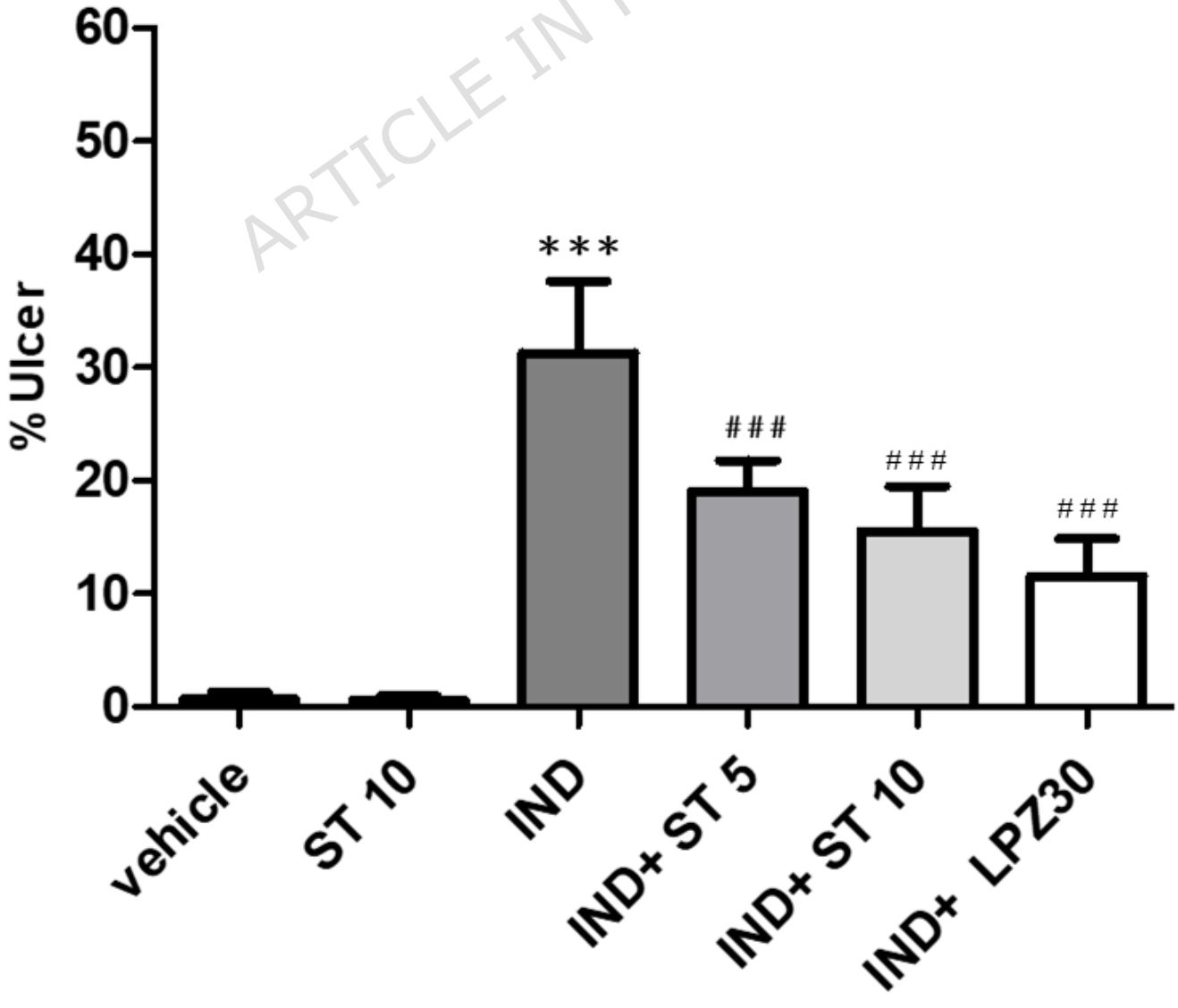

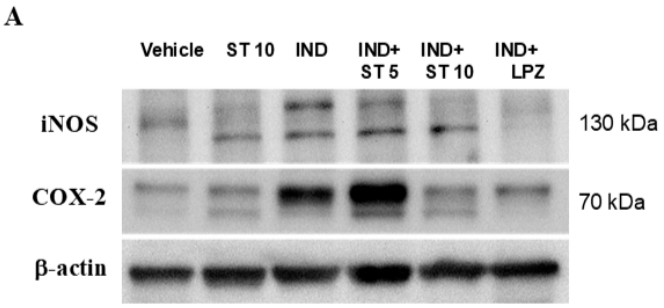

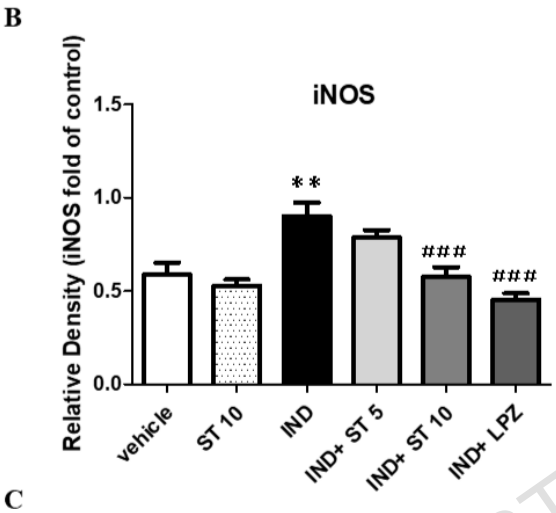

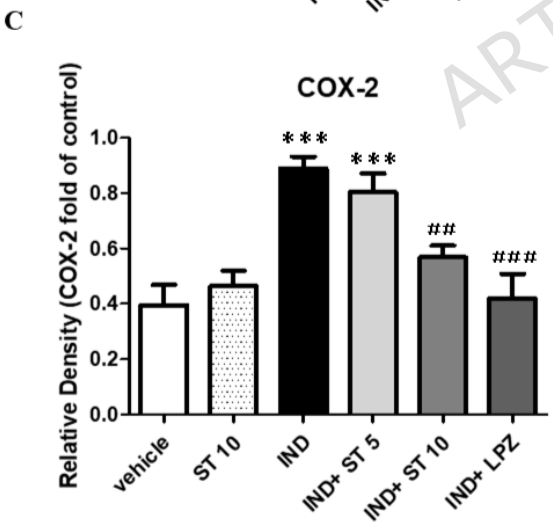

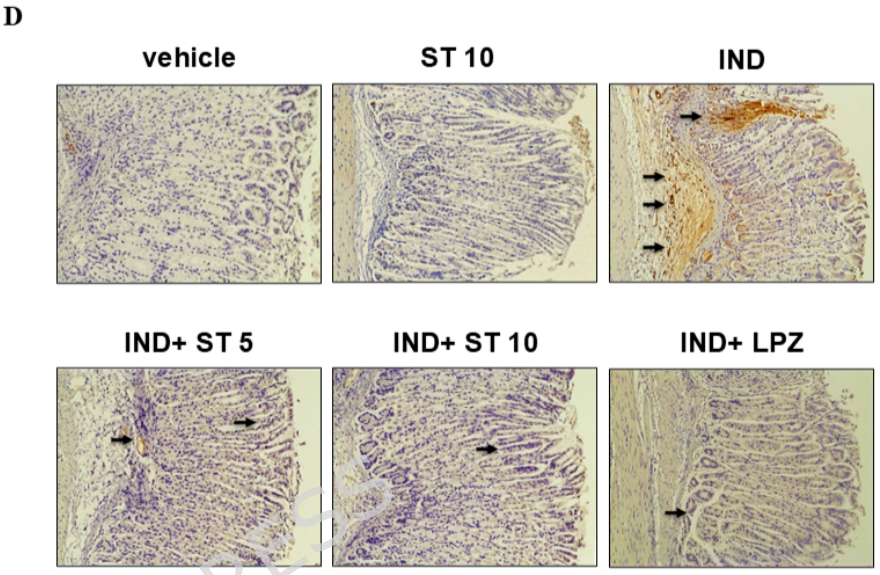

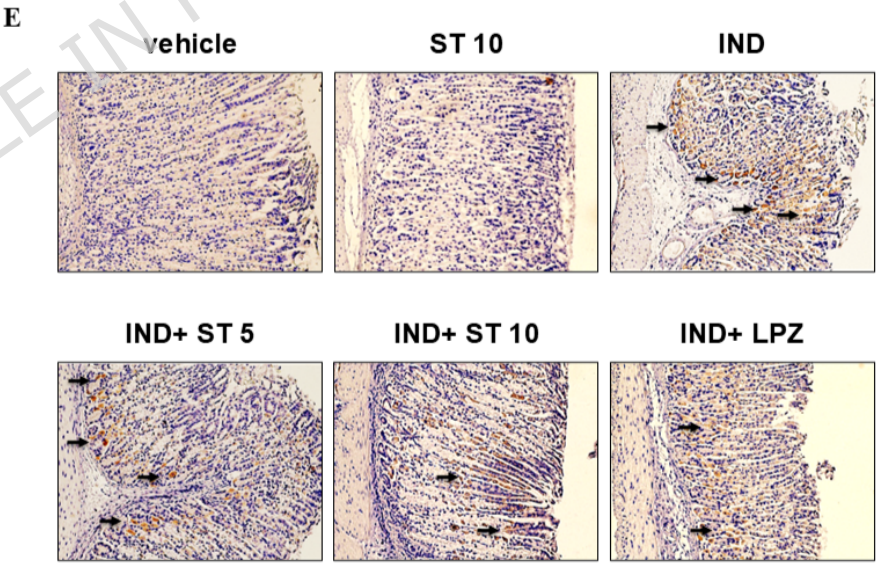

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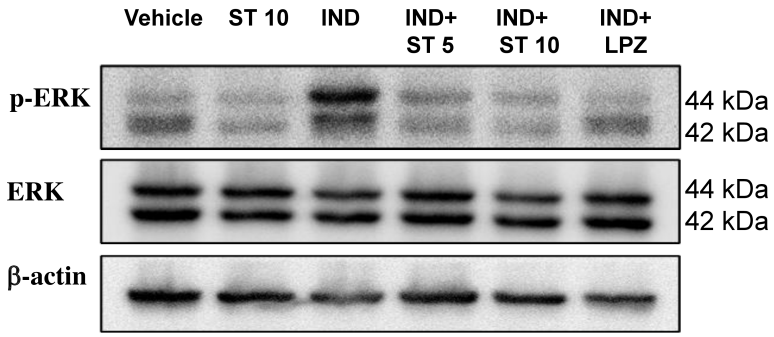

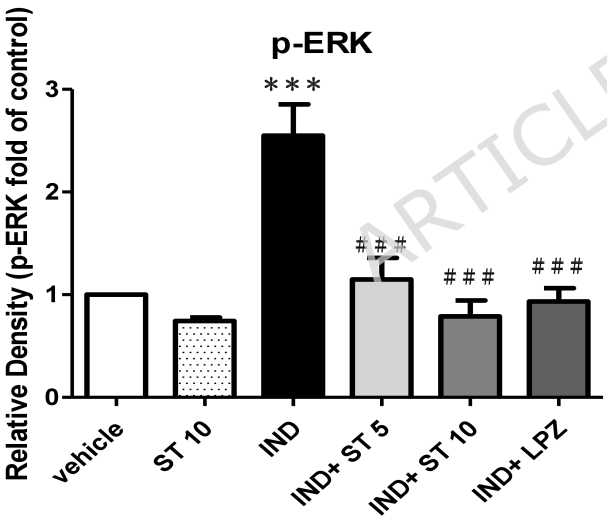

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