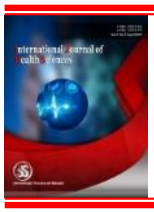

International Journal of Health Sciences

Available online at www.sciencescholar.us
Vol. 7 No. 3, December 2023, pages: 95-107
e-ISSN: 2550-696X, p-ISSN: 2550-6978
https://doi.org/10.53730/ijhs.v7n3.14624

Anti-Tumor Effect of Fasudil, a ROCK Inhibitor, on Gastric Cancer in Mice

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Manuscript submitted: 18 July 2023, Manuscript revised: 09 September 2023, Accepted for publication: 27 October 2023

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Keywords

apoptosis;
Fasudil;
gastric cancer;
inflammation;
oxidative stress;

Abstract

Gastric cancer remains one of the most common malignant tumors and a leading cause of death. However, there are few reports about the anti-tumor effect of Fasudil (Fas) on the stomach. Establishment of nude mice model of gastric cancer to evaluate the anti-tumor effect of Fas. The mice in each group were weighed every week. Serum and tumor tissue cytokines are detected by commercial kits. Serum and tumor tissue superoxide dismutase and malondialdehyde were detected by commercial kits. Histopathological changes in the tumor were detected by HE staining. Expression of ROS/ TXNIP/NLRP3/Caspase-1 pathway in tumor tissues was detected by Western blot. Our results showed that Fas increased and decreased the body weight of tumor mice, increased serum and tumor tissue cytokines contents of tumor mice, increased serum and tumor tissue oxidative stress of tumor mice, and increased Bax, Caspase-3, Caspase-9 protein expression, reduced Bcl-2 protein expression, also regulated ROS/TXNIP/NLRP3/Caspase-1 pathway expression. Our experiments show that Fas has a significant anti-gastric cancer effect, and its mechanism is related to the regulation of oxidative stress, inflammation and apoptosis in vivo.

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1 Introduction

Stomach cancer is one of the most common cancers and the fourth leading cause of cancer-related deaths (Sung et al., 2021). Due to the lack of early detection, most patients with gastric cancer are diagnosed at an advanced stage and have a poor prognosis (Smyth et al., 2020). In the past decades, despite great efforts to improve the diagnosis and treatment of gastric cancer, the prognosis for many gastric cancer patients remains dismal, with a mortality rate of gastric cancer as high as 8.2 cases per 100,000 people, suggesting that there is an urgent need for innovative approaches to combat this malignant tumor (Thrift & El-Serag, 2020). In recent years, increasing attention has been paid to the development of novel therapeutic strategies, including the repurposing of existing drugs, to improve the therapeutic efficacy of gastric cancer.

Fasudil is an effective and selective inhibitor of Rhokinase (ROCK), ROCK is an effector of the small guanosine triphosphate enzyme Rho (Wang et al., 2022). It performs a variety of cellular functions, including smooth muscle contraction, actin cytoskeleton tissue, cell adhesion, and fine cell migration (Lv et al., 2022). At present fasudil has been widely used in temporary bed therapy for spasmodic cerebral blood vessels in China and Japan (Tanaka et al., 2005), which can relieve spinal cord injury, stroke, Parkinson's disease, neuropathic pain and epilepsy caused by central nervous system disorders. In addition, studies have reported that fasudil has anti-tumor effect and may inhibit the growth of breast cancer cells (Guerra et al., 2017), glioblastoma cells (Deng et al., 2010) and laryngeal cancer cells (Zhang & Wu, 2018). However, the mechanism of fasudil's inhibitory effect on gastric cancer is still unclear. The purpose of this article is to study the mechanism of fasudil's inhibitory effect on gastric cancer.

2 Materials and Methods

Reagents

Fas was purchased from the Dalian Meilun Biotechnology Co., ltd. RPMI-1640 culture solution was purchased from Gibco Company in the United States, and fetal bovine serum and double antibody were both purchased from Thermophilic Technology Company. IL-1β, IL-6 and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were produced by Nanjing KeyGen Biotech. Co., Ltd. All antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA).

Cell line and culture

A549 cells were purchased from the Cell Resource Center of the Shanghai Institute of Life Sciences, Chinese Academy of Sciences. It was placed in a cell culture solution (DUL BECCO' S Modified Eagle' S Medium (DMEM) containing 10% fetal bovine serum and 0.01 mg/mL bovine insulin, and cultured in a 5% CO2 incubator at 37°C, and logarithmic growth cells were taken for experiments (Macias-Alvia et al., 2022).

Establishment of tumor animal model

Gastric cancer BGC-823 cells were collected at a concentration of 1 $\times 10^8$/ml, and 75% alcohol was prepared for axillary disinfection in nude mice. 0.2 ml (cell number 2×10^7) tumor cell suspension was injected subcutaneously into each nude mouse. Tumors developed after 1 week and reached a diameter of about 0.8-1 cm after 2 weeks. Tumor mice were divided into three groups: model group, 5-fluorouracil group (5-

Fu,10mg/kg), Fas (5mg/kg), 5-fluorouracil group and Fas group were given gavage administration for 28 days respectively, and the model group was given physiological saline of corresponding volume.

Establishment of body mass change curve and tumor volume change curve of nude mice

Since the establishment of the tumor-bearing mice model, the weight of mice was weighed every 3 d; The tumor volume was measured and recorded every 3 days since Fas was fed by self-irrigation. Note: tumor volume calculation formula, tumor volume =ab2/2(a is length, b is width). GraphPad Prism 5 was used to draw the mass change curve and tumor volume change curve of nude mice.

Collection and treatment of tumor specimens

The tumor-bearing mice in each group were killed by neck removal method on 29th d. Tumor specimens were taken immediately to observe tumor morphology, measure tumor diameter and calculate tumor volume. Then some tumor tissues were cut and fixed in 4% paraformaldehyde, and the remaining specimens were frozen at-80°C for later use.

Determination of inflammatory cytokines in serum and tumor tissue

The levels of IL-6, IL-1β and TNF-α in serum and tumor tissue were measured by ELISA kits according to the manufacturer's instructions. The protein contents of these samples were detected with a BCA kit and normalized to the data of biochemical parameters.

Determination of ROS tumor tissue

ROS levels in liver tissue were detected with the fluorescence probe 2′ 7′-dichlorodihydrofluorescein diacetate (DCFH2-DA). For ROS assay, tumor tissue was homogenized in saline solution and centrifuged at 10,000 × g for 15 min at 4 °C to get the supernatants. Five micromole probe DCFH2-DA (dissolved in PBS) was added into the supernatants at 37 °C for 20 min. Fluorescence was measured at λex = 488 nM and λem= 525 nm with a microplate reader (Thermo Scientific, Schwerte, Germany). The protein contents of liver samples were detected with a BCA protein assay kit and normalized to the data of ROS levels.

Histological examination of tumor tissue

Tumor tissue fixed with 4% paraformaldehyde was embedded in paraffin to prepare 4 μm slices, which were operated according to the routine procedure of HE staining.

Immunohistochemical detection

Specimens fixed with 4% paraformaldehyde solution were embedded in conventional paraffin and made into 4 μm thick sections, which were operated according to the instructions of the immunohistochemistry kit. Image-Pro Plus 6. 0 was used to analyze the results absorbance (A Value) value for statistical analysis.

Western blot

The tumor tissue frozen at-80°C was added with protein lysate and crushed for 1 min by ultrasonic pulverizer, then cracked on ice for 30 min, centrifuged at 12 000 r/min at 4°C for 10 min, and the supernatant was taken as total protein. The concentration of protein was determined by the BCA method, 50 μg of the sample was added to each sample by adding a hole, the protein was separated by SDS-PAGE, and 70 V electrophoresis was used. The film was transferred at 4°C and 250 mA constant current. The blocking solution was blocked at 37°C for 2 h. Primary antibodies were used for incubation overnight at 4°C, secondary antibody was incubated at 37°C for 2 h, TBST was rinsed, ECL reagent was illuminated, and Bio-Rad illumination system was imaged.

Bio-Rad Image-Lab-Software was used to analyze and determine the bands (Russo et al., 2006; Doonan, & Cotter, 2008).

Statistical Analysis

SPSS 22. 0 software was used for statistical analysis. The experimental data were expressed by the mean standard deviation (means ± SDs) and the mean comparison of the two samples was conducted by T-test.

3 Results and Discussions

3.1 Results

Effect of Fas on changes in body mass of experimental mice

The tumor volume of Fas-fed mice was significantly smaller than that of control mice (Figure. 1A). The weight of mice in the Fas group showed a gradually increasing trend while that of mice in the control group showed a gradually decreasing trend with the increase of tumor volume (Figure. 1B).

Effect of Fas on tumor volume curve

The tumor-bearing mice were killed and stripped of the tumor. The tumor in the control group was indistinct from the surrounding tissues, difficult to strip and easy to bleed, and the tumor section was grey and white. In the Fas gavage group, the tumor infiltrating into the surrounding tissues is light and easy to peel off, and the tumor section is greyish-red, grey, and dull, scattered in necrotic foci of different sizes. The tumor diameter was measured (Figure. 1C) and the tumor volume was calculated. It was found that the tumor volume of the Fas perfusion group was significantly smaller than that of the model group (Figure. 1D).

Effect of Fas on cytokines in serum

As shown in Figure. 2A, compared with the model group, Fas increased cytokines in serum of tumor-bearing nude mice. The results showed that Fas could improve the inflammatory response of tumor-bearing nude mice.

Effect of Fas on cytokines in tumor tissue

As shown in Figure. 2B, compared with the model group, Fas increased cytokines in tumor tissue of tumor-bearing nude mice. The results showed that Fas could improve the inflammatory response of tumor-bearing nude mice.

HE staining

In the model group, the tumor cells were abundant and disorderly arranged, the cells were polygonal, the nuclei were significantly heteromorphic, and had more pathological mitotic figures, less nuclear shrinkage, and a smaller area of sheet necrosis (Yamashita et al., 2007; Wang et al., 2011). The tumors in the Fas group tumor cells in the tissue were spindle-shaped and round, with cell shrinkage, deep nucleus staining, and significantly reduced mitotic figures, showing scattered focal and fused necrosis (Figure. 2C).

Effect of Fas on ROS level in tumor tissue
As shown in Figure. 3A, As expected, compared with the model group, Fas significantly increased the level of ROS in serum.

Effect of Fas on ROS/TXNIP/NLRP3/Caspase-1 in tumor tissue

The levels of Txnip, NLRP3, Caspase-1, and IL-1β of the Fas group were significantly increased compared to the model group, and the expression levels of Trx were significantly decreased in the Fas group than in the model group (Figure. 3B).

Effect of Fas on Apoptosis related protein in tumor tissue

As shown in Figure. 4, compared with the model group, western blot showed that the protein levels of Bax, Caspase-3 and Caspase-9 increased in the Fas group at different concentrations while Bcl-2 decreased.

3.2 Discussion

Gastric cancer is a malignant tumor originating from the epithelium of the gastric mucosa, whose early symptoms are atypical and easily missed, thus increasing the difficulty of early diagnosis (Sies, 2018). In addition, the clinical manifestations of gastric cancer are relatively complicated, and most patients are in the middle and late stages when they are diagnosed, thus losing the best time for treatment, so the prognosis is poor. Due to various serious side effects of chemotherapy and its long treatment cycle, it is difficult for patients to persist in completing treatment. External radiation radiotherapy requires a high dose, which will increase irreversible damage to normal gastric tissue and further damage gastric function. Therefore, we urgently need to find effective drugs to treat gastric cancer (Saif et al., 2010). Our results show that Fas increased and decreased the body weight of tumor mice, increased serum and tumor tissue cytokines contents of tumor mice, increased serum and tumor tissue oxidative stress of tumor mice, and increased Bax, Caspase-3, Caspase-9 protein expression, reduced Bcl-2 protein expression, also regulated ROS/TXNIP/NLRP3/Caspase-1 pathway expression (Sarkar & Fisher, 2006; Ravizza et al., 2011).

Continuous activation of inflammasome corpuscle can cause overexpression of cytokines, leading to the occurrence of chronic inflammation and autoimmune diseases. Inflammation and immunity play an important role in the occurrence of gastric cancer. Nucleotide binding oligodomain receptor (NLR) plays an important role in the composition of inflammatory corpuscles. For example, Backert et al (Pachathundikandi & Backert, 2018) found that activation of the NLRP3 pathway promoted the secretion of the anti-inflammatory factor IL-10 from primary gastric cancer cells in co-culture. The NLRP3 inflammasome is currently one of the most extensively studied inflammasomes, consisting of the NLRP3 receptor, ASC, and caspase-1. Activation of the inflammasome leads to the maturation and extracellular release of pro-inflammatory cytokines like IL-1β and IL-18, thus regulating the immune response (Broz & Dixit, 2016). Additionally, the inflammasome can trigger a cascade resulting in pyroptosis, a pro-inflammatory form of programmed cell death with similarities to necrosis and apoptosis, dependent on the activation of caspase-1 or caspase-11 (Jiang et al., 2018). The activation of the inflammasome is governed by two types of signals. The primary signal involves the activation of NF-κB, which induces the upregulation of NLRP3, IL-1β precursor, and IL-18 precursor in cells. Secondary signals that activate the NLRP3 inflammasome include the generation of ROS from mitochondrial disruption, cathepsin, the efflux of anions and osmolytes via VRAC, and the influx of Ca2+. Oligomerization and activation of the NLRP3 inflammasome result in the cleavage of pro-IL-1β and pro-IL-18 by caspase-1, leading to the production and release of mature forms of IL-1β and IL-18, thereby initiating inflammation (Freeman et al., 2020). Previous research has indicated that prolonged chronic inflammation can cause tissue damage, thereby promoting tumor progression. Key molecules in various inflammasome pathways, such as IL-1β, IL-18, caspase-1, and ASC, have been confirmed to participate in the tumorigenesis and progression (Wang et al., 2018).

4 Conclusion

In this study, compared with the model group, the level of ROS in tumor tissue was decreased. As expected, Fas significantly reduced the level of ROS in tumor tissue. The levels of Txnip, NLRP3, Caspase-1, and IL-1β of

the Fas group were significantly increased compared to the model group, and the levels of Trx were significantly decreased in the Fas group than in the model group. Fas also restored apoptosis related protein in tumor tissue.

To sum up, Fas inhibited gastric cancer, and its molecular mechanism may provide new theoretical basis for Fas to treat gastric cancer by regulating ROS/TXNIP/NLRP3/Caspase-1 signal pathway, thus widening the pharmacological effects of Fas. This study is only limited to in vivo studies, and the next step will be in vitro experiments to further prove fasudil's anticancer potential.

Acknowledgments
We are grateful to two anonymous reviewers for their valuable comments on the earlier version of this paper.

References

Broz, P., & Dixit, V. M. (2016). Inflammasomes: mechanism of assembly, regulation and signalling. *Nature Reviews Immunology*, *16*(7), 407-420.

Deng, L., Li, G., Li, R., Liu, Q., He, Q., & Zhang, J. (2010). Rho-kinase inhibitor, fasudil, suppresses glioblastoma cell line progression in vitro and in vivo. *Cancer biology & therapy*, *9*(11), 875-884.

Doonan, F., & Cotter, T. G. (2008). Morphological assessment of apoptosis. *Methods*, *44*(3), 200-204. https://doi.org/10.1016/j.ymeth.2007.11.006

Freeman, S., Brough, D., & El-Sharkawy, L. (2020). Inhibiting the NLRP3 Inflammasome. *Molecules*, *25*(5533).

Guerra, F. S., Oliveira, R. G. D., Fraga, C. A. M., Mermelstein, C. D. S., & Fernandes, P. D. (2017). ROCK inhibition with Fasudil induces beta-catenin nuclear translocation and inhibits cell migration of MDA-MB 231 human breast cancer cells. *Scientific reports*, *7*(1), 13723.

Jiang, D., Chen, S., Sun, R., Zhang, X., & Wang, D. (2018). The NLRP3 inflammasome: role in metabolic disorders and regulation by metabolic pathways. *Cancer letters*, *419*, 8-19. https://doi.org/10.1016/j.canlet.2018.01.034

Lv, Z., Ding, Y., Cao, W., Wang, S., & Gao, K. (2022). Role of RHO family interacting cell polarization regulators (RIPORs) in health and disease: Recent advances and prospects. *International Journal of Biological Sciences*, *18*(2), 800.

Macias-Alvia, A. M., Zambrano-Santos, R. O., Zambrano-Ubillus, R. P., & Ubillús-Saltos, S. (2022). Self-care in primary health care in the nursing career. *International Journal of Health Sciences*, *6*(3), 1671–1684. https://doi.org/10.53730/ijhs.v6n3.13694

Pachathundikandi, S. K., & Backert, S. (2018). Helicobacter pylori controls NLRP3 expression by regulating hsa-miR-223-3p and IL-10 in cultured and primary human immune cells. *Innate immunity*, *24*(1), 11-23.

Ravizza, T., Balosso, S., & Vezzani, A. (2011). Inflammation and prevention of epileptogenesis. *Neuroscience letters*, *497*(3), 223-230. https://doi.org/10.1016/j.neulet.2011.02.040

Russo, A., Terrasi, M., Agnese, V., Santini, D., & Bazan, V. (2006). Apoptosis: a relevant tool for anticancer therapy. *Annals of Oncology*, *17*, vii115-vii123. https://doi.org/10.1093/annonc/mdl963

Saif, M. W., Makrilia, N., Zalonis, A., Merikas, M., & Syrigos, K. (2010). Gastric cancer in the elderly: an overview. *European Journal of Surgical Oncology (EJSO)*, *36*(8), 709-717. https://doi.org/10.1016/j.ejso.2010.05.023

Sarkar, D., & Fisher, P. B. (2006). Molecular mechanisms of aging-associated inflammation. *Cancer letters*, *236*(1), 13-23. https://doi.org/10.1016/j.canlet.2005.04.009

Sies, H. (2018). On the history of oxidative stress: Concept and some aspects of current development. *Current Opinion in Toxicology*, *7*, 122-126. https://doi.org/10.1016/j.cotox.2018.01.002

Smyth, E. C., Nilsson, M., Grabsch, H. I., van Grieken, N. C., & Lordick, F. (2020). Gastric cancer. *The Lancet*, *396*(10251), 635-648.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians*, *71*(3), 209-249.

Tanaka, K., Minami, H., Kota, M., Kuwamura, K., & Kohmura, E. (2005). Treatment of cerebral vasospasm with intra-arterial fasudil hydrochloride. *Neurosurgery*, *56*(2), 214-223.

Thrift, A. P., & El-Serag, H. B. (2020). Burden of gastric cancer. *Clinical Gastroenterology and Hepatology*, *18*(3), 534-542. https://doi.org/10.1016/j.cgh.2019.07.045

Wang, H., Luo, Q., Feng, X., Zhang, R., Li, J., & Chen, F. (2018). NLRP3 promotes tumor growth and metastasis in human oral squamous cell carcinoma. *BMC cancer*, *18*, 1-10.

Wang, N., Guan, P., Zhang, J. P., Chang, Y. Z., Gu, L. J., Hao, F. K., ... & Chu, L. (2011). Preventive effects of fasudil on adriamycin-induced cardiomyopathy: possible involvement of inhibition of RhoA/ROCK pathway. *Food and chemical toxicology*, *49*(11), 2975-2982. https://doi.org/10.1016/j.fct.2011.06.080

Wang, Q., Song, L. J., Ding, Z. B., Chai, Z., Yu, J. Z., Xiao, B. G., & Ma, C. G. (2022). Advantages of Rho-associated kinases and their inhibitor fasudil for the treatment of neurodegenerative diseases. *Neural regeneration research*, *17*(12), 2623.

Yamashita, K., Kotani, Y., Nakajima, Y., Shimazawa, M., Yoshimura, S. I., Nakashima, S., ... & Hara, H. (2007). Fasudil, a Rho kinase (ROCK) inhibitor, protects against ischemic neuronal damage in vitro and in vivo by acting directly on neurons. *Brain research*, *1154*, 215-224. https://doi.org/10.1016/j.brainres.2007.04.013

Zhang, X., & Wu, N. (2018). Fasudil inhibits proliferation and migration of Hep-2 laryngeal carcinoma cells. *Drug design, development and therapy*, 373-381.

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Figure legends

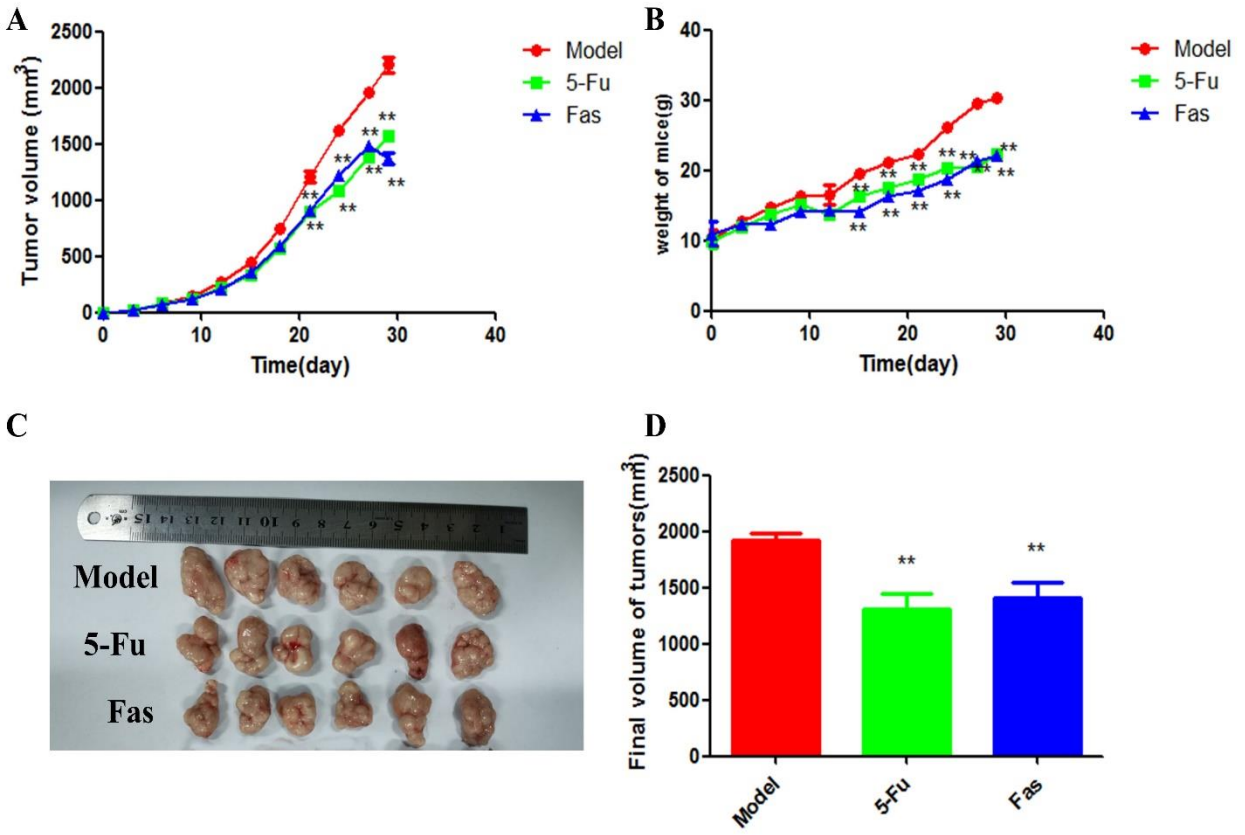

Figure 1. Effect of Fas on (A) tumor volume and (B) weight change, (C) tumor diameter and (D) final tumor volume in experimental mice

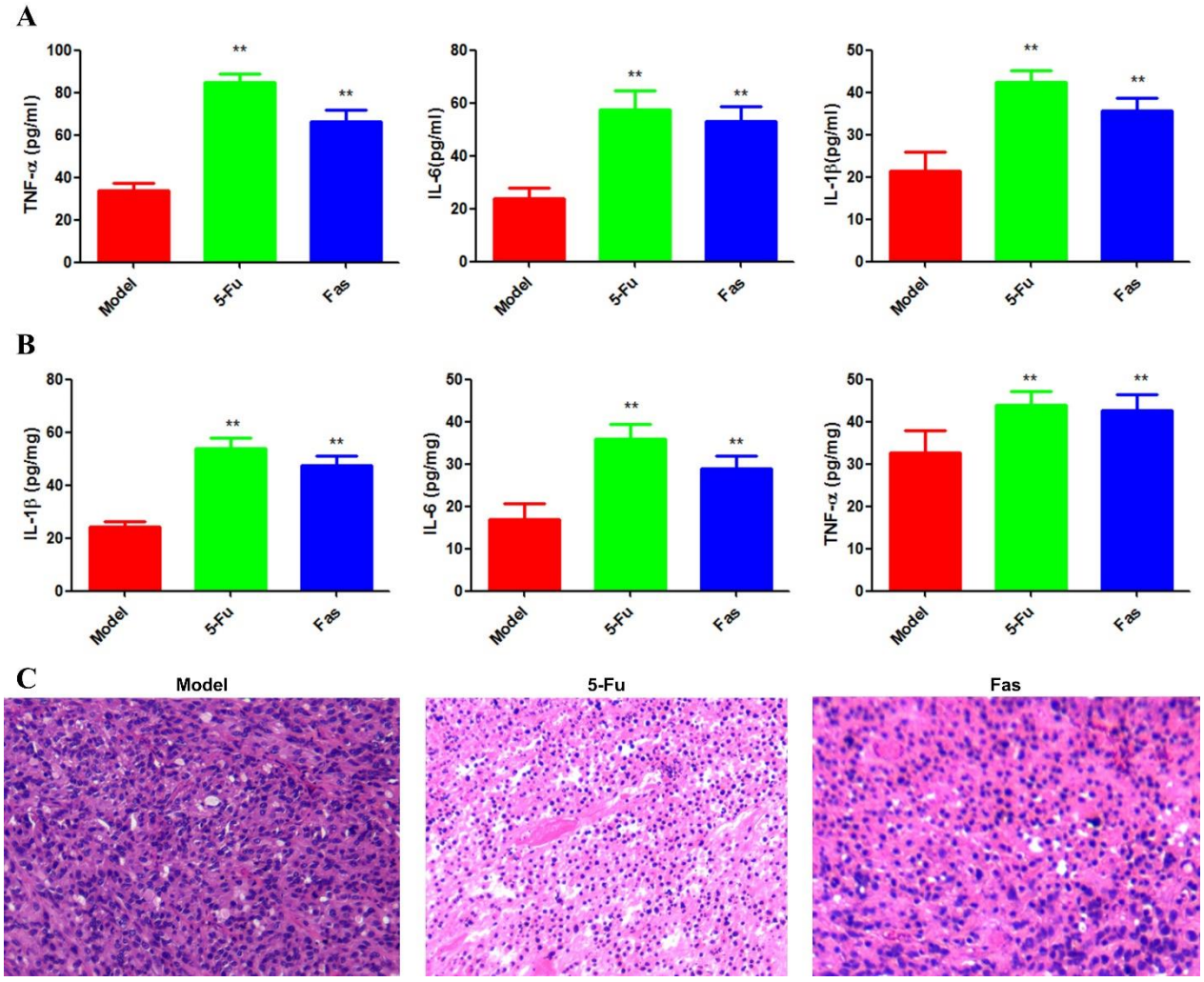

Figure 2. Effect of Fas on cytokines in (A) serum, (B) tumor tissue, and (C) HE staining

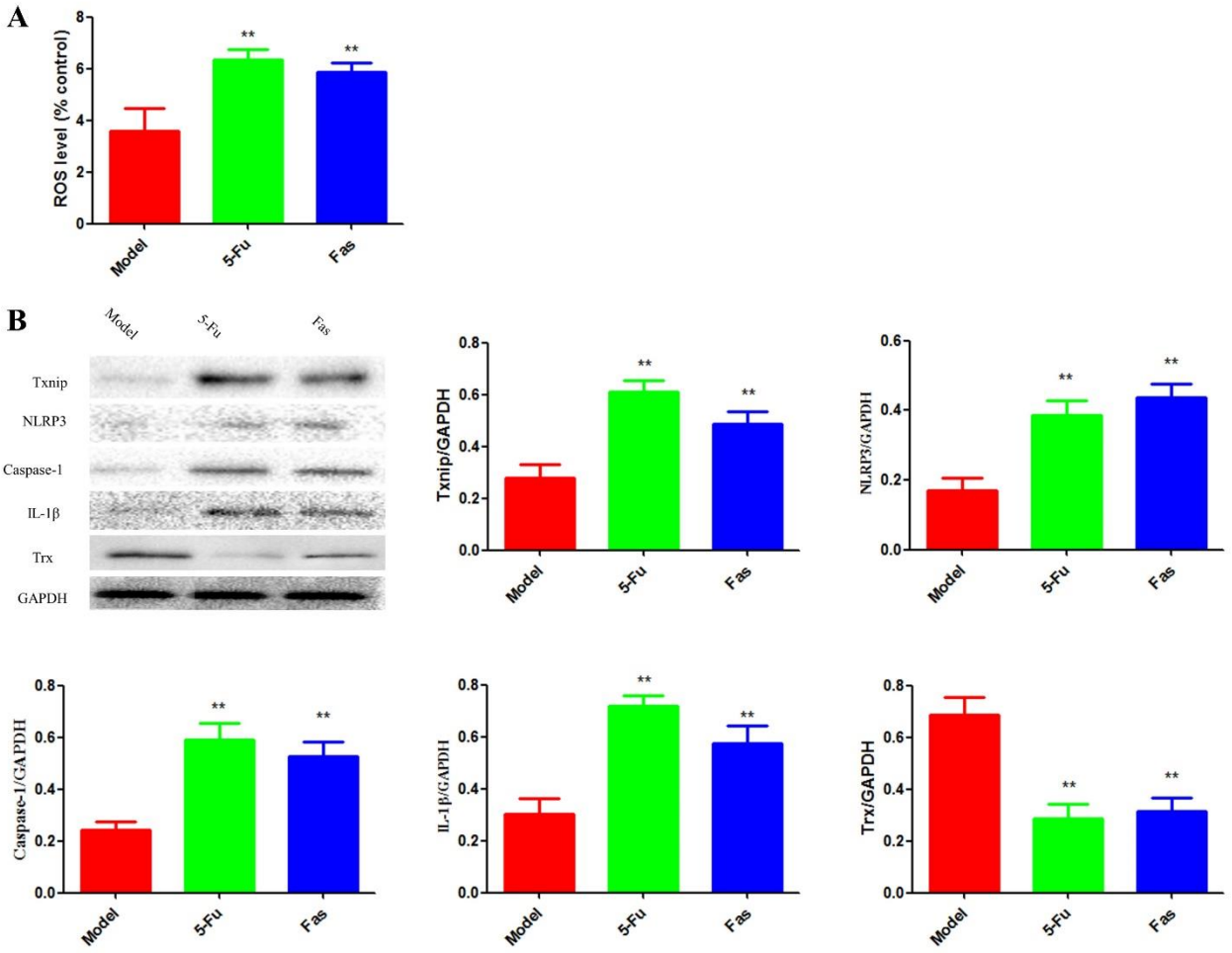

Figure 3. Effect of Fas on ROS/TXNIP/NLRP3/Caspase-1 in tumor tissue

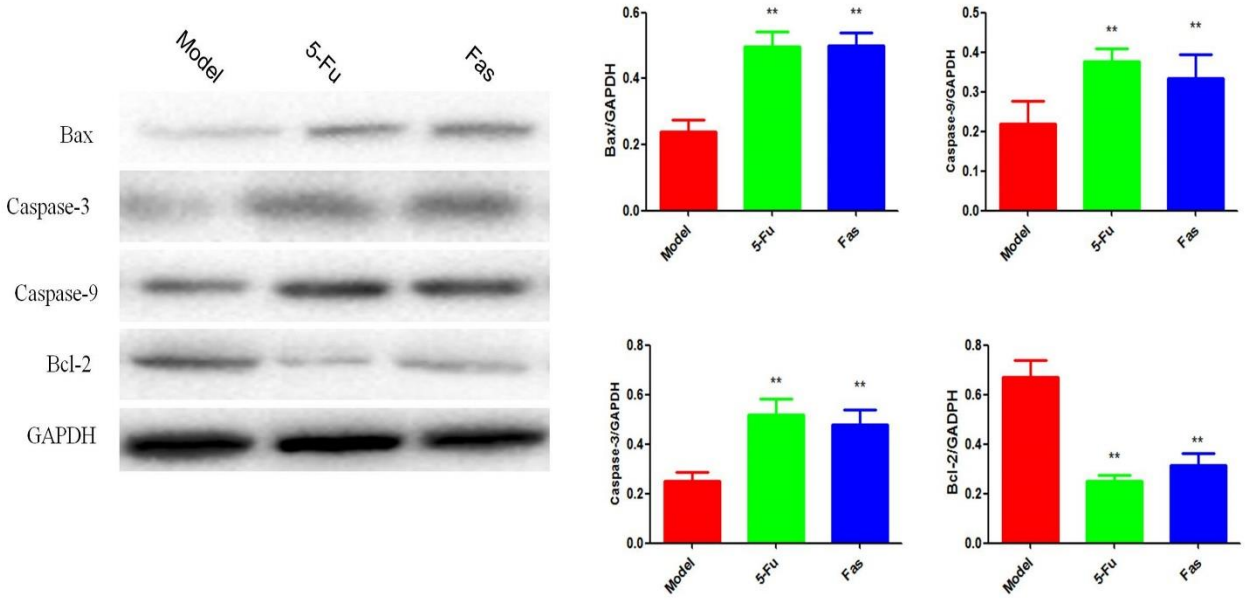

Figure 4. Effect of Fas on Apoptosis related protein in tumor tissue