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Research article

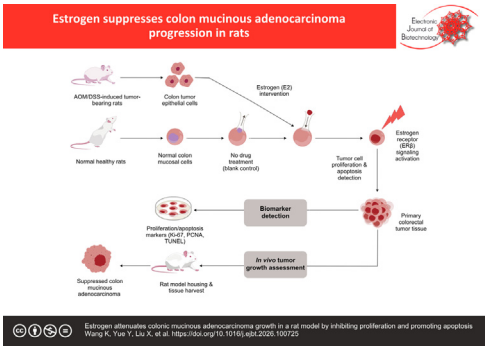
Estrogen attenuates colonic mucinous adenocarcinoma growth in a rat model by inhibiting proliferation and promoting apoptosis ☆

Ke Wang [a], Yumin Yue [a], Xiaoli Liu [b], Meng Fan [b,*]

[a] Department of Gastrointestinal Surgery, Xianyang Central Hospital, Xi'an, Shaanxi Province, China
[b] Department of Gynecology, Xi'an Fengcheng Hospital, Xi'an, Shaanxi Province, China

GRAPHICAL ABSTRACT

Estrogen attenuates colonic mucinous adenocarcinoma growth in a rat model by inhibiting proliferation and promoting apoptosis.



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ABSTRACT

Background: Colonic mucinous adenocarcinoma is a highly aggressive subtype of colon cancer. Epidemiological evidence has linked estrogen exposure to certain gastrointestinal malignancies, yet its pathophysiological role in the pathogenesis of colonic mucinous adenocarcinoma remains unclear. This study aimed to investigate the effect of estrogen on tumor growth in a rat model of colonic mucinous adenocarcinoma and its association with intestinal mucosal epithelial proliferation-related factors and apoptosis.

Results: Compared with the normal control group, the model control group showed significantly increased tumor proliferative activity and elevated intestinal epithelial apoptosis. After estrogen intervention, tumor volume and proliferative activity were inhibited, the expressions of Ki-67 and proliferating cell nuclear antigen were downregulated, and the apoptosis rate of intestinal epithelial cells decreased; an estrogen antagonist could enhance some of the above effects.

Conclusions: Estrogen can slow the growth of colonic mucinous adenocarcinoma by inhibiting intestinal mucosal epithelial proliferation, exhibiting potential anti-tumor effects. Its mechanism may be related to regulating the proliferation-apoptosis balance of intestinal mucosal epithelium, providing experimental evidence for colon cancer therapy research.

☆ Audio abstract available in Supplementary material.

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* Corresponding author.
E-mail addresses: 230240250260270@finmail.com, lilya0806@163.com (M. Fan).

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1. Background

Colon mucinous adenocarcinoma is recognized as a highly aggressive subtype of colon cancer, characterized by tumor cells secreting copious amounts of mucus. This results in a loose tumor tissue structure, which subsequently enhances its invasive and metastatic potential [1,2]. Compared with other types of colon cancer, early diagnosis of colon mucinous adenocarcinoma is particularly challenging, and the development of effective treatment strategies is often constrained [3,4]. Numerous studies have confirmed that this subtype is associated with a poor prognosis and high recurrence rates, positioning it as a critical focus in colorectal cancer research aimed at elucidating pathogenic mechanisms and developing novel therapeutic targets [5,6]. A recent systematic review and meta-analysis demonstrated that patients with colon mucinous adenocarcinoma have a significantly elevated risk of mortality, 44% higher than those with non-mucinous adenocarcinoma. This adverse prognostic effect is even more pronounced in patients with colon cancer and those with stage IV advanced disease [7], further underscoring the urgent need for targeted therapeutic research specifically for this distinct subtype.

The development and progression of colon cancer result from an interplay of genetic susceptibility, environmental factors, and endocrine factors [8]. Among these, estrogen, a key endocrine hormone, is closely implicated in the pathogenesis of various tumors [9,10]. Estrogen regulates cellular proliferation, differentiation, and apoptosis by binding to estrogen receptors, a mechanism believed to play a significant role in colorectal tumorigenesis [11,12]. However, its specific pathophysiological role in colon mucinous adenocarcinoma remains unclear. Currently, surgical resection remains the cornerstone of clinical treatment for colorectal cancer, supplemented by systemic therapies such as chemotherapy and targeted therapy. Although the management of metastatic colorectal cancer has advanced over recent decades, with median overall survival increasing from 12 months during 1986–1996 to 21 months during 2007–2015, these improvements have been largely incremental. The 5-year survival rate remains low at 12.2%, and in most clinical trials, the improvement in overall survival attributable to new drugs is less than 2 months, with a concurrent decline in the novelty of therapeutic targets [13]. The limitations of traditional treatment modalities, coupled with the unique malignant biological characteristics of colon mucinous adenocarcinoma, make the discovery of novel therapeutic targets and regulatory mechanisms crucial for improving patient outcomes in this subtype.

Ki-67 and proliferating cell nuclear antigen (PCNA) are established markers for assessing the proliferative activity of intestinal mucosal epithelial cells. Ki-67 primarily labels cells in the active phases of the cell cycle [14,15], while PCNA is a characteristic molecule expressed during DNA synthesis. Both markers provide a direct reflection of the degree of cellular proliferation [16,17], and detecting their expression levels can offer valuable insights into the proliferative activity of intestinal tumors. Previous studies have suggested that estrogen participates in colon carcinogenesis by modulating the intestinal microenvironment and influencing the expression of cell cycle- and proliferation-related factors [18,19]. Nevertheless, this effect has not been rigorously validated in colon mucinous adenocarcinoma, and the specific relationship between estrogen and proliferation-related factors in the intestinal mucosa epithelium warrants further experimental investigation.

Notably, the malignant features of colon mucinous adenocarcinoma are closely linked to the stemness characteristics of cancer stem cells. This stemness not only underpins the high invasiveness and metastatic potential of this subtype but also constitutes a core mechanism underlying treatment resistance. Signaling pathways such as nuclear factor-kappa B (NF-κB) play a pivotal role in regulating the stemness of colorectal cancer stem cells and shaping the malignant phenotype of the disease. Furthermore, the choice of anesthetic technique during colorectal cancer surgery has been shown to correlate with oncological outcomes. Immunosuppression resulting from perioperative anesthesia strategies can impact the patient's immune status. For instance, combined general anesthesia with epidural analgesia has been demonstrated to significantly enhance postoperative levels of natural killer cells and CD4+ T cells in patients undergoing colon cancer surgery, whereas the effects of intravenous versus inhalational anesthesia on immune parameters remain inconclusive [20]. The potential modulatory effects of local anesthetics on the stem-like characteristics of colon cancer cells also present a new avenue for perioperative intervention and target discovery in colorectal cancer.

In summary, this study utilizes a DSS/AOM-induced rat model of colon mucinous adenocarcinoma to investigate the influence of estrogen on tumor growth in this specific subtype. It further analyzes the association of estrogen with intestinal mucosal epithelial proliferation-related factors (Ki-67, PCNA) and apoptosis (TUNEL). The objective is to elucidate the role and potential mechanisms of estrogen in colon mucinous adenocarcinoma, thereby providing experimental evidence for estrogen receptor-targeted therapy in this subtype. This research also aims to offer theoretical support for developing novel therapeutic strategies for colorectal cancer, aligning with the pressing need for new therapeutic targets and mechanistic insights within the current colorectal cancer research landscape.

2. Materials and methods

2.1. Experimental animals and grouping

Female Wistar rats, weighing 180–220 g and aged 8–10 weeks, were purchased from Beijing Beoyu Biotechnology Co., Ltd. The rats were acclimatized for 7 d in a controlled environment (temperature: 22 ± 2°C; humidity: 50 ± 10%; 12 h light/dark cycle), during which they had free access to standard laboratory chow and tap water.

Rats were randomly divided into groups using a random number table method, with 15 rats per group. Random numbers were generated using SPSS 27.0, and grouping was performed according to the range of values to ensure baseline body weight and physical condition were balanced across groups. A strict blinding method was implemented throughout the experiment: personnel responsible for animal feeding, drug administration, and tumor monitoring were blinded to group allocation; personnel responsible for pathological section evaluation, immunohistochemical analysis, and statistical analysis were also blinded to group allocation. Unblinding was performed only after all experimental data had been collected.

The primary measure was the Ki-67 positive rate in the colon mucinous adenocarcinoma model. Based on pre-experimental results, the expected difference between groups was set at 15%, with a significance level of $\alpha = 0.05$ and a statistical power of $1-\beta = 0.8$. The sample size was calculated using one-way ANOVA, yielding a minimum sample size of 10 rats per group. Considering potential animal exclusion due to modeling failure or drug reactions, as well as the high heterogeneity of the colon cancer model, 15 rats were assigned to each group to ensure statistical power, effectively avoid false-negative results associated with small sample sizes, and accommodate the heterogeneity of the model.

The groups were as follows: normal control group (NC group): no treatment; model control group (MC group): DSS/AOM modeling was performed [21], with no intervention; estrogen group (E2 group): after modeling, rats received weekly intraperitoneal injections of 17β-estradiol 2 mg/kg (Cat# 228703, Beijing Bailingwei Technology Co., Ltd.) for 8 weeks. The dosage was determined based on a study by Li et al. [22], which showed that this dose significantly regulated intestinal epithelial cell proliferation; estrogen + antagonist group (E2+ICI group): in addition to the same treatment as the E2 group, rats received daily intraperitoneal injections of ICI 182780 10 mg/kg (Cat# XY-00029, Shanghai Xinyu Biotechnology Co., Ltd.) for 8 weeks. The dosage was determined by pre-experiments to effectively antagonize estrogen receptor activity.

All surgical procedures were performed under anesthesia induced by intraperitoneal injection of ketamine hydrochloride 75 mg/kg (2 mL: 0.1 g, Shanxi Taiyuan Pharmaceutical Co., Ltd., National Drug Approval Number H14022824). Postoperatively, rats were given ibuprofen 5 mg/kg (4 mL: 0.4 g, Jilin Sihuan Pharmaceutical Co., Ltd., National Drug Approval Number H20203061) for analgesia to alleviate postoperative discomfort.

2.2. Establishment of the tumor model in rats

After 7 d of acclimatization, rats received an intraperitoneal injection of azoxymethane (AOM, 12 mg/kg, Sigma-Aldrich) on d 1. Two weeks later, 2.5% dextran sulfate sodium (DSS, molecular weight: 36–50 kDa, MP Biomedicals) was administered in drinking water for 7 d, followed by a 14-d recovery period with regular drinking water. This DSS treatment cycle was repeated twice, with a total modeling duration of 8 weeks.

Criteria for successful modeling and heterogeneity control measures: Following surgical exposure of the colon, tumor tissues with a diameter > 0.5 cm were excised. Tissues were identified as colon mucinous adenocarcinoma if the mucin content exceeded 50%, as determined by Alcian blue staining. Only rats with successful modeling were included in subsequent experiments. To minimize model heterogeneity, the same batches of AOM and DSS reagents were used throughout the experiment. The injection volume and pushing speed of AOM were strictly controlled. DSS drinking water was freshly prepared and used immediately, and water intake was recorded for each rat to ensure consistent DSS consumption. All rats were housed in a uniform environment during the modeling period.

Tumor growth was monitored by abdominal palpation after surgery to verify successful modeling (Fig. 1).

During the surgical procedure, one rat in the MC group developed a severe anesthetic reaction with respiratory depression and did not complete the AOM injection; therefore, it was excluded. Another rat in the MC group failed to develop tumors due to inaccurate administration during DSS/AOM modeling, where the drug was injected into the colonic lumen instead of the abdominal cavity; thus, it was excluded due to modeling failure. Consequently, 13 rats in the MC group were successfully modeled. In the E2 group, one rat exhibited a significant allergic reaction, characterized by skin rash and respiratory distress, following the first intraperitoneal injection of estrogen. This was determined to be drug intolerance, and the rat was excluded and euthanized by cervical dislocation. Ultimately, 14 rats remained in the E2 group. In the E2+ICI group, one rat developed local induration and inflammation at the injection site during continuous administration. This was confirmed to be due to incomplete drug absorption, resulting in unstable plasma concentrations and unreliable experimental data. The rat was excluded and euthanized by cervical dislocation. Ultimately, 14 rats remained in the E2+ICI group.

Fig. 1. The abdominal swelling (or protrusion) of the rats.

2.3. Monitoring of tumor growth

Tumor growth was monitored weekly by abdominal palpation combined with color Doppler ultrasound (RS-N50, Xuzhou RuishengChaoying Technology Co., Ltd.) to avoid detection bias introduced by palpation alone. For ultrasound monitoring, rats were anesthetized with a low dose of ketamine hydrochloride (30 mg/kg), and abdominal hair was removed. Ultrasound gel was applied to the transducer, and the colon tumor site was scanned. Tumor images were captured, and the three perpendicular diameters (length a, width b, height c) of the tumor were measured using the built-in measurement software of the ultrasound device, with a measurement accuracy of 0.01 cm. Abdominal palpation was performed by the same trained investigator using standardized measurement criteria to record tumor size, minimizing operator-induced error. During tumor growth, body weight was measured every two weeks using an electronic balance (accuracy 0.01 g) to record weight changes and assess the impact of the tumor on the overall health of the rats. The tumor volume was calculated using [Equation 1]:

$$V = \frac{\pi}{6} \times a \times b \times c \quad (1)$$

The three-dimensional tumor diameters measured by ultrasound were substituted into the formula for quantitative volume calculation, and tumor growth curves were plotted based on weekly tumor volume data.

2.4. Immunohistochemical staining

At the end of the experiment, all rats were euthanized by cervical dislocation and dissected. The colon was removed, and the tumor site was carefully examined. The colon and tumor tissues were separated, washed to remove excess blood and impurities, and preserved in PBS buffer at 4°C. Subsequently, the tissues were fixed in 10% formalin solution for 24 h. The fixed tissue samples

were dehydrated, cleared, and embedded in paraffin without damaging their morphology. Finally, the fixed and embedded tissues were cut into slices with a thickness of 5 μm using a microtome and transferred to glass slides.

Tissue sections were first deparaffinized using xylene, followed by rehydration through a graded ethanol series (100% ethanol twice for 10 min each, 95% ethanol for 5 min, and 75% ethanol for 5 min). Antigen retrieval was performed by immersing the sections in 0.01 M citrate buffer (pH 6.0) and incubating in a water bath at 80°C for 30 min. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide for 15 min. Sections were then incubated with primary antibodies [Ki-67 (1:200, KBR-HS2024, Shanghai Keborui Biotechnology Co., Ltd.) and PCNA (1:100, V15618329298, Shanghai Yuduo Biotechnology Co., Ltd.)] for 2 h at room temperature, followed by overnight incubation at 4°C. The next day, HRP-conjugated anti-mouse secondary antibody (1:500, ATB02007H, Wuhan Antebo Technology Co., Ltd.) was applied and incubated at 37°C for 30 min. After DAB chromogenic development, sections were observed under a microscope (Olympus SZX10).

Apoptotic cells were detected using the TUNEL assay kit (Roche). After proteinase K digestion of tissue sections, TdT enzyme and biotin-labeled dUTP were added, followed by incubation at 37°C for 1 h. After DAB chromogenic development, apoptotic cells with brownish-yellow nuclei were counted to calculate the positive rate.

Western blot analysis was performed to detect ERα and ERβ expression. Tumor tissues were lysed with RIPA buffer to extract total protein, and protein concentration was determined using the BCA assay. Equal amounts of protein were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. Membranes were incubated overnight at 4°C with primary antibodies against ERα (1:1000, Abcam ab32063) and ERβ (1:1000, BES1006K, BIOESN). Subsequently, membranes were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5000, Jackson ImmunoResearch Laboratories) at room temperature for 1 h. Protein bands were visualized by ECL chemiluminescence, and densitometric analysis was performed using *Image Lab*, with β-actin serving as the loading control.

2.5. Histopathological analysis

Rat intestinal and tumor tissue sections (5 μm thick) were immersed in hematoxylin solution for 10 min, with hematoxylin binding to DNA in the cell nucleus to produce a blue-purple color. Subsequently, the sections were immersed in eosin solution for 5 min, with eosin staining the cytoplasm, extracellular matrix, and other components, resulting in pink or red cytoplasm. After hydration, the sections were dehydrated through a gradient of ethanol (75–95–100%), washed with xylene, and finally mounted with a mounting gel. Systematic microscopic evaluation was performed to record the morphological changes in the intestinal structure and tumor tissue.

2.6. Observation indicators

(1) ***Intestinal tissue structure.***

The assessment of intestinal tissue structure was based on HE-stained sections, focusing on the arrangement of intestinal epithelial cells, glandular structure, and the integrity of the intestinal tissue. The scoring criteria are as follows.

Normal structure (0 points): Epithelial cells are neatly arranged, glandular structures are clear, nuclei are blue-purple, cytoplasm is pink, and glands are orderly arranged.

Mild damage (1 point): Mild hyperplasia or slight disorder in the arrangement of epithelial cells, with recognizable glandular structures.

Moderate damage (2 points): Visible epithelial hyperplasia, disordered glandular structures, enlarged glandular lumens, and the appearance of cellular atypia.

Severe damage (3 points): Severe destruction of the epithelial layer, glandular disintegration, visible hyperplasia of intestinal epithelial cells, and the presence of ulcers or ruptured intestinal glands.

(2) ***Tumor growth characteristics and morphological changes.***

The size, shape, tissue type, invasiveness, and differentiation degree of the tumor were observed after HE staining to evaluate the growth and malignancy of the tumor. The scoring criteria are as follows.

Tumor size: Small (<0.5 cm, score 0); medium (0.5–1.5 cm, score 1); large (>1.5 cm, score 2).

Tumor tissue morphology: Uniform glandular structure, well-differentiated, and regularly arranged cells (score 0); irregular glandular morphology with some tumor cells showing high atypia (score 1); highly irregular tumor cells with no distinct glandular structure, extremely irregular cell morphology, and features of malignant tumors (score 2).

Tumor invasiveness: No invasion (score 0); invasion into surrounding tissues or local lymph nodes (score 1); extensive tumor invasion into adjacent tissues and metastasis formation (score 2).

(3) ***Expression of proliferation-related factors in intestinal epithelium (Ki-67 and PCNA).***

The expression of proliferation-related factors Ki-67 and PCNA in the intestinal epithelium was assessed by IHC.

(4) ***Intestinal apoptosis.***

The assessment of intestinal apoptosis was performed using the TUNEL staining method. TUNEL staining marks apoptotic cells, and the proportion of positive cells is calculated to evaluate the level of apoptosis.

(5) ***The body weight and food consumption of the rats were recorded weekly. The average daily food intake was calculated using an electronic balance (accuracy 0.1 g).***

(6) ***Quantitative analysis of immunohistochemically positive areas.***

Quantitative analysis of immunohistochemical staining images was performed using ImageJ software to calculate the area of positive regions and assess the expression levels of proliferation-related factors.

2.7. Statistical analysis

All experimental data were analyzed using SPSS 27.0 statistical software. The Shapiro-Wilk test was adopted to determine whether the data followed a normal distribution. For data that were normally distributed, one-way ANOVA was adopted to compare distinctions among groups, and if the results were significant ($p < 0.05$), further Tukey's HSD tests were conducted; for comparisons between two groups, an independent-samples *t*-test was adopted. Data were represented as mean ± SD, with statistical significance set at $p < 0.05$. Pearson correlation coefficients were adopted to assess the linear relationships between different variables.

3. Results

3.1. Contrast of intestinal tissue structure

In Fig. 2, the NC group exhibited normal intestinal structure with a score of 0. The MC group showed severe intestinal structural damage with a score of 2.4 ± 0.6, visibly higher than the NC group; the intestinal structural damage in the E2 group and E2+ICI group was improved compared with the MC group, with scores of 1.8 ± 0.5 and 1.3 ± 0.4, respectively ($p < 0.05$).

In Fig. 3, HE staining (×200) of intestinal tissues from each group revealed normal epithelium in the NC group, glandular disorder and mucus lakes with crypt destruction in the MC group, glandular repair in the E2 group, and reduced mucus in the E2 +ICI group.

3.2. Changes in body weight and food consumption

In Table 1, after model establishment, the MC group exhibited decreased body weight and reduced food intake due to tumor burden (81% and 23% lower than the NC group, respectively, $p < 0.05$). Estrogen therapy (E2 group) improved body weight and food intake, whereas estrogen antagonism (E2+ICI group) reversed this improvement, suggesting that estrogen may alleviate tumor-induced cachexia by modulating metabolism.

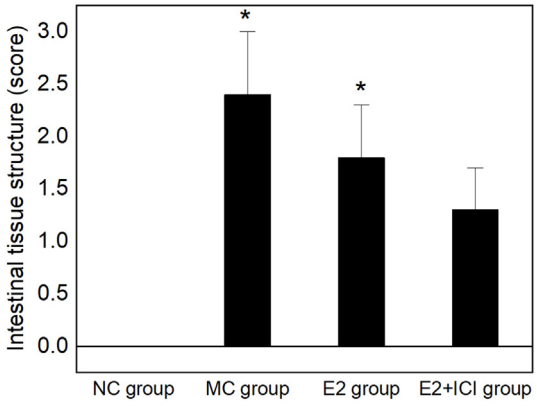

Fig. 2. Contrast of intestinal tissue structure. Note: *indicates a visible distinction compared with the NC group ($p < 0.05$). NC group: n = 15; MC group: n = 13; E2 group: n = 14; E2+ICI group: n = 14.

3.3. Contrast of tumor growth characteristics and morphological changes

In Fig. 4, the MC group exhibited severe tumor size, tissue morphology, and invasiveness, with scores of 2, 2, and 2, respectively, visibly higher than the NC group (all scores were 0). The tumor size and tissue morphology in the E2 group and E2+ICI group were improved, with scores of 1.5 ± 0.5 and 1.7 ± 0.6, respectively. The tumor invasiveness in the E2 group and E2+ICI group was visibly reduced, with scores of 1.3 ± 0.4 and 1.1 ± 0.3 ($p < 0.05$).

3.4. Contrast of expression levels of intestinal epithelial proliferation-related factors

In Fig. 5, the proportions of Ki-67 and PCNA positive cells in the MC group were visibly increased, with Ki-67 being 45.3 ± 4.3 and PCNA being 43.2 ± 5.6, much higher than those in the NC group (10.2 ± 2.1 and 9.5 ± 1.9); the proliferative activity in the E2 group and the E2+ICI group was inhibited compared with the MC group, with Ki-67 being 35.6 ± 3.8 and 28.1 ± 2.4, and PCNA being 33.1 ± 4.2 and 26.3 ± 3.1 ($p < 0.05$).

Fig. 6 shows Ki-67 immunohistochemical staining. The NC group exhibited a small number of brown-positive cells at the crypt base, while the MC group displayed numerous deeply stained brown positive cells throughout the entire epithelial layer. The E2 group showed significantly fewer positive cells, mainly confined to the crypt base. In contrast, the E2+ICI group had increased positive cells compared to the E2 group, with their distribution extending from the crypt base to the mid-crypt region.

Fig. 7 shows the PCNA immunohistochemical staining. In the NC group, scattered weakly stained brown positive cells were observed at the crypt base. The MC group exhibited densely distributed dark brown positive cells throughout the entire epithelial layer. The E2 group showed reduced positive cell numbers and weaker staining intensity, primarily localized at the crypt base. In contrast, the E2+ICI group displayed increased positive cell numbers and staining intensity compared to the E2 group, with positive cells visible in the mid-crypt region.

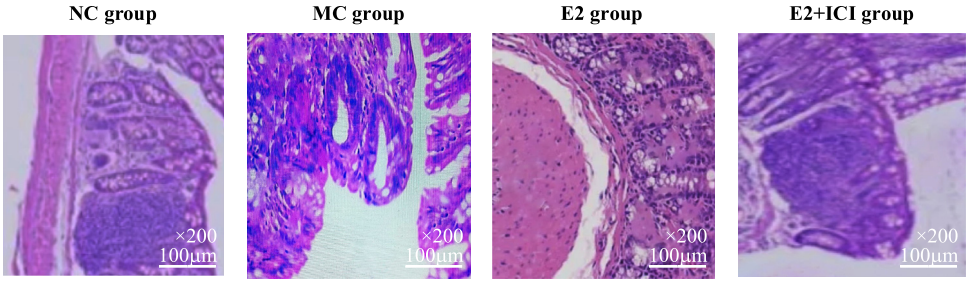

Fig. 3. Histopathology and tumor morphology by HE staining (×200).

Table 1
Results of body weight and food consumption.

Group	Initial weight (g)	Weight at week 8 (g)	Weight change (g)	Daily average food intake (g/d)
NC group (n = 15)	195 ± 10	258 ± 12	+63 ± 8	18.5 ± 1.2
MC group (n = 13)	198 ± 8	210 ± 15*	+12 ± 5*	14.2 ± 1.5*
E2 group (n = 14)	192 ± 9	235 ± 10#	+43 ± 7#	16.8 ± 1.3#
E2+ICI group (n = 14)	196 ± 7	215 ± 13*	+19 ± 6*	14.5 ± 1.4*

Note: weight change: MC and E2+ICI group showed significant decreases compared to NC group (*$p < 0.05$), while E2 group showed significant increase compared to MC group (#$p < 0.05$); Food intake: MC and E2+ICI group showed significant decreases compared to NC group (*$p < 0.05$), while E2 group showed significant increase compared to MC group (#$p < 0.05$).

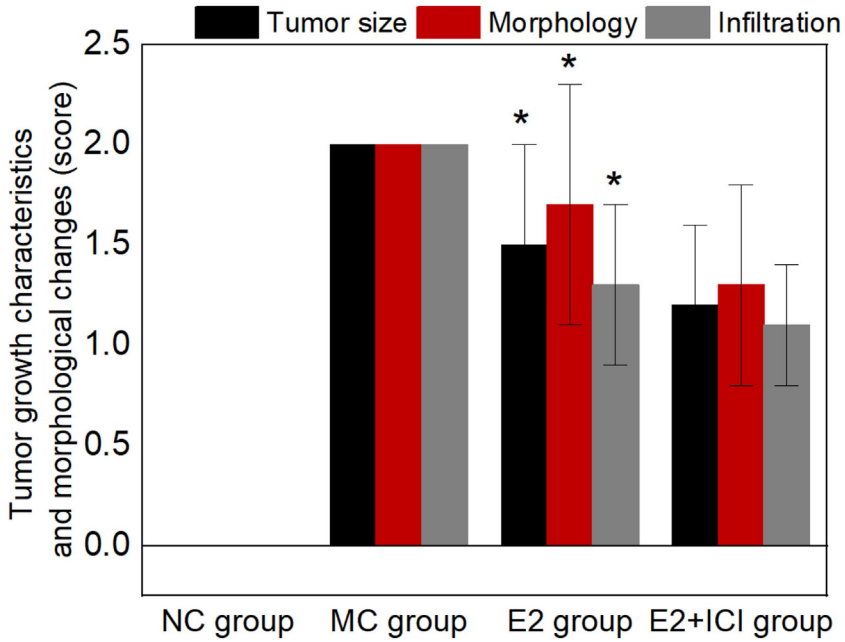

Fig. 4. Contrast of tumor growth characteristics and morphological changes. Note: *indicates against the NC group, $p < 0.05$. NC group: n = 15; MC group: n = 13; E2 group: n = 14; E2+ICI group: n = 14.

3.5. Estrogen receptor expression levels

In Table 2, ERβ expression in tumor tissues of the model group (MC) was significantly higher than that in the normal group (NC) (2.3-fold, $p < 0.05$), while ERα expression showed a mild increase. Estrogen treatment (E2 group) further upregulated ERβ expression (52% increase compared to the MC group, $p < 0.05$), whereas estrogen antagonism (E2+ICI group) reversed this elevation, suggesting that estrogen may exert its effects through ERβ.

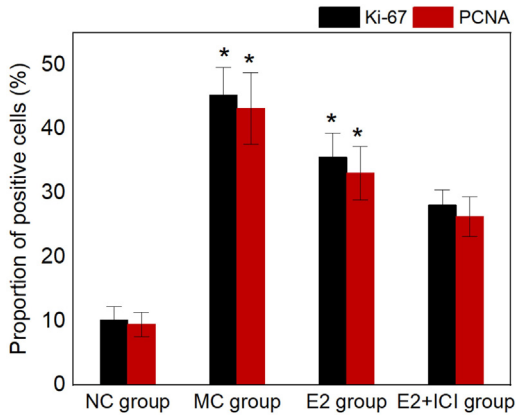

Fig. 5. Contrast of expression levels of intestinal epithelial proliferation-related factors. Note: * indicates against the NC group, $p < 0.05$. NC group: n = 15; MC group: n = 13; E2 group: n = 14; E2+ICI group: n = 14.

3.6. Contrast of intestinal apoptosis

Fig. 8 shows that the proportion of apoptotic intestinal epithelial cells was significantly increased in the MC group, with the percentage of TUNEL-positive cells being 8.9 ± 1.1, which was significantly higher than that in the NC group (2.1 ± 0.5, $p < 0.05$), suggesting that the occurrence and development of colon mucinous adenocarcinoma were accompanied by an elevated level of intestinal epithelial cell apoptosis. The apoptotic proportions in the E2 group and the E2+ICI group were lower than those in the MC group, at 6.7 ± 0.8 and 5.1 ± 0.7, respectively, and both were statistically significantly different from the MC group ($p < 0.05$), indicating that estrogen intervention could reduce the level of intestinal epithelial cell apoptosis in rats with colon mucinous adenocarcinoma, and this effect was more pronounced when combined with an estrogen antagonist.

3.7. Quantitative analysis of immunohistochemically positive areas

In Fig. 9, the Ki-67 and PCNA positive areas in the MC group were visibly increased, with Ki-67 being 22.4 ± 3.3 and PCNA being 21.1 ± 3.5, visibly higher than those in the NC group (6.2 ± 1.1 and

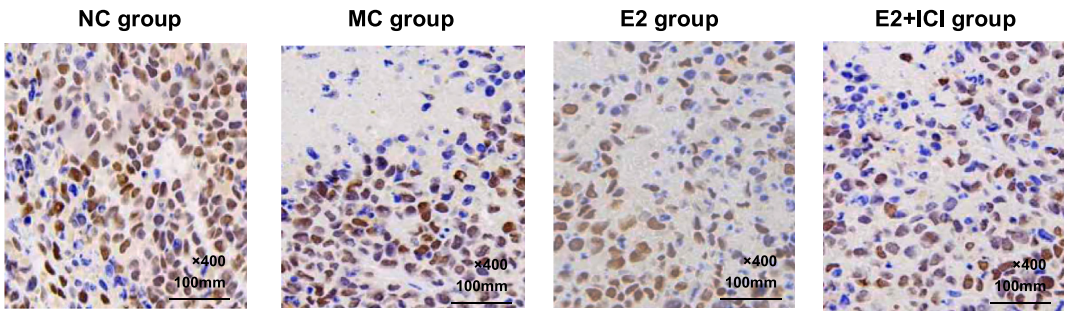

Fig. 6. Ki-67 immunohistochemical staining (×400).

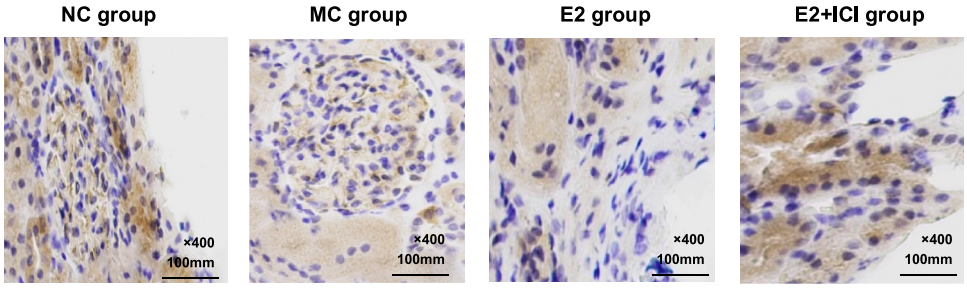

Fig. 7. PCNA immunohistochemical staining (×400).

Table 2
Western blot analysis of ERα and ERβ expression.

Group	ERα/β-actin (Gray value ratio)	ERβ/β-actin (Gray value ratio)
NC group (n = 15)	0.12 ± 0.03	0.35 ± 0.05
MC group (n = 13)	0.25 ± 0.04*	0.82 ± 0.08*
E2 group (n = 14)	0.18 ± 0.03#	1.25 ± 0.10#
E2+ICI group (n = 14)	0.24 ± 0.05*	0.79 ± 0.07*

Note: ANOVA: ERα ($F = 12.6$, $p < 0.001$), ERβ ($F = 21.3$, $p < 0.001$). Tukey's HSD: Both ERα and ERβ in the MC group were significantly higher than the NC group (*$p < 0.05$); ERβ in the E2 group was significantly higher than the MC group (#$p < 0.05$), while ERα showed no significant difference; ERβ in the E2+ICI group was significantly lower than the E2 group (*$p < 0.05$), approaching MC group levels.

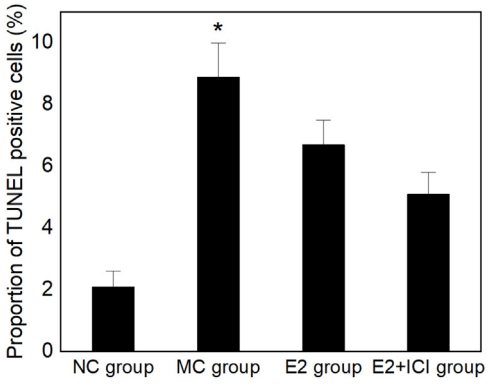

Fig. 8. Contrast of intestinal apoptosis. Note: *Statistically significant difference compared with the NC group ($p < 0.05$); #Statistically significant difference compared with the MC group ($p < 0.05$). NC group, n = 15; MC group, n = 13; E2 group, n = 14; E2+ICI group, n = 14.

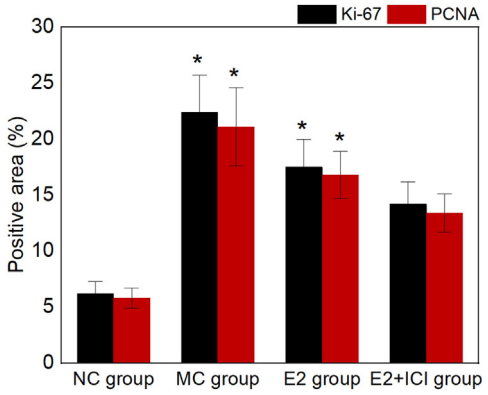

Fig. 9. Quantitative analysis of immunohistochemically positive areas. Note: *indicates against the NC group, $p < 0.05$. NC group: n = 15; MC group: n = 13; E2 group: n = 14; E2+ICI group: n = 14.

5.8 ± 0.9); the positive areas in the E2 group and the E2+ICI group were smaller, with Ki-67 being 17.5 ± 2.5 and 14.2 ± 2.0, and PCNA being 16.8 ± 2.1 and 13.4 ± 1.7 ($p < 0.05$).

3.8. Correlation analysis

Table 3 shows the correlation coefficients (r values) and significance (p values) between the variables. Ki-67 and PCNA expression were highly positively correlated ($r = 0.92$), indicating a very strong linear relationship between these two multiplication markers in intestinal epithelial multiplication. Ki-67 was strongly positively correlated with tumor size ($r = 0.84$), indicating a close relationship between the multiplication of intestinal epithelial cells and tumor size. PCNA was moderately positively correlated with tumor size ($r = 0.80$), indicating a certain positive correlation between multiplication activity and tumor size. The proportion of TUNEL-positive cells was moderately positively correlated with tumor size ($r = 0.55$), suggesting that when the tumor was larger, apoptosis was more pronounced. Ki-67 was negatively correlated with the proportion of TUNEL-positive cells ($r = -0.60$), indicating that when multiplication was active, the level of apoptosis may be lower.

3.9. Limitations of mechanistic investigation in this study

This experiment only examined the expression levels of estrogen receptor subtypes ERα and ERβ in tumor tissues, confirming that ERβ may be the primary receptor mediating estrogen regulation in colon mucinous adenocarcinoma. However, the following limitations regarding the mechanistic investigation remain: Upstream and downstream signaling nodes of ERβ (e.g., the PI3K/Akt and MAPK/ERK signaling pathways) were not examined, preventing the identification of the specific signal transduction pathways through which ERβ exerts its regulatory effects. Further experiments are required for validation. Cancer stem cell-like markers (e.g., CD133, Lgr5) and NF-κB downstream factors (e.g., TNF-α, IL-6) in tumor tissues were not assessed, leaving the association between estrogen intervention and tumor stemness or inflammatory signaling pathways unclear. Furthermore, the transcriptional regulation of the proliferation-apoptosis axis was not analyzed in depth. The specific molecular mechanisms by which estrogen inhibits intestinal epithelial proliferation and regulates apoptosis warrant further investigation using molecular biology techniques such as transcriptome sequencing.

4. Discussion

This study employed a rat model of colon mucinous adenocarcinoma induced by DSS/AOM to investigate the regulatory effect of estrogen on tumor growth, while also analyzing its association with intestinal mucosal epithelial proliferation-related factors and cell apoptosis. The results confirmed that estrogen effectively

Table 3
Results of correlation analysis.

Variables comparison	Pearson correlation coefficient (r)	p	Strength of correlation
Ki-67 vs. PCNA expression	0.92	<0.001	Highly positive correlation
Ki-67 vs. tumor size	0.84	<0.01	Strong positive correlation
PCNA vs. tumor size	0.8	<0.05	Moderate positive correlation
Proportion of TUNEL-positive cells vs. tumor size	0.55	<0.05	Moderate positive correlation
Ki-67 vs. proportion of TUNEL-positive cells	−0.6	<0.05	Moderate negative correlation

ameliorated intestinal tissue structural damage in the model rats, significantly inhibited tumor proliferative activity, and slowed the process of malignant invasion. Furthermore, this effect was primarily mediated through the upregulation of ERβ receptor expression in tumor tissues. The intestinal tissue damage score in the model control group reached 2.4 ± 0.6, which was significantly higher than that in the normal control group, confirming that DSS/AOM modeling successfully induced colon mucinous adenocarcinoma and caused severe damage to the intestinal mucosa. However, following estrogen intervention, the intestinal tissue damage scores in the estrogen group and the estrogen + antagonist group decreased to 1.8 ± 0.5 and 1.3 ± 0.4, respectively, suggesting that estrogen alleviated intestinal mucosal damage by regulating local intestinal immune responses and epithelial repair mechanisms [23], thereby exerting a protective effect on intestinal tissue and subsequently delaying the occurrence and progression of colon tumors. Regarding tumor growth characteristics, the model control group exhibited scores of 2 for tumor size, histomorphology, and invasiveness, displaying typical malignant biological features. In contrast, after estrogen intervention, the malignancy-related tumor scores in each group were significantly reduced, indicating that estrogen inhibited invasive tumor growth by modulating immune responses in the tumor microenvironment, cell cycle progression, and apoptotic signaling pathways [24], demonstrating a potential anti-colon mucinous adenocarcinoma effect.

Immunohistochemical and quantitative analysis results showed that the positivity rates and positive areas of Ki-67 and PCNA in intestinal epithelial cells were significantly increased in the model control group, with Ki-67 reaching 45.3 ± 4.3 and PCNA reaching 43.2 ± 5.6, much higher than those in the normal control group, confirming that the occurrence and development of colon mucinous adenocarcinoma were accompanied by abnormally enhanced proliferative activity of intestinal epithelial cells. In contrast, the expression of proliferation-related factors and positive areas was significantly downregulated in the estrogen group and the estrogen + antagonist group, indicating that estrogen exerted its tumor-suppressive effect by directly inhibiting the proliferative activity of intestinal mucosal epithelial cells. Activation of estrogen receptors can influence the proliferation and differentiation processes of intestinal epithelial cells, thereby inhibiting the occurrence and development of intestinal tumors to a certain extent [25]. At the level of apoptosis, the proportion of TUNEL-positive cells in the model control group was 8.9 ± 1.1, which was significantly higher than that in the normal control group, suggesting that apoptotic processes were involved in the tumor development of colon mucinous adenocarcinoma, possibly related to the compensatory clearance of tumor cells by the organism and mechanisms of tumor immune escape. However, after estrogen intervention, the apoptotic proportion in rat intestinal epithelial cells showed a clear decreasing trend. This result initially appeared logically inconsistent with the tumor-suppressive effect of estrogen but reflected the cell specificity and tumor microenvironment dependence of estrogen's regulation of tumor growth. It is speculated that the regulatory effect of estrogen on colon mucinous adenocarcinoma is not a singular pro-proliferative or pro-apoptotic effect, and its core action on tumor cells is proliferation inhibition, while the mild downregulation of apoptosis is a secondary effect. On one hand, estrogen may reduce abnormal apoptosis of normal intestinal epithelial cells by regulating the tumor microenvironment, maintaining the structural and functional integrity of the intestinal mucosa, thereby weakening the histological basis for tumorigenesis. On the other hand, the regulatory effect of estrogen depends on the expression proportion of the ERβ receptor in tumor tissues. In this study, ERβ expression was significantly increased in the model control group and was further upregulated after estrogen intervention, and this effect could be reversed by the antagonist, suggesting that, as the main receptor through which estrogen exerts its effects, the expression level of ERβ directly influences the regulatory outcome of estrogen on the proliferation-apoptosis balance. This receptor-dependent regulatory pattern also led to the asynchronous changes in proliferation and apoptosis indicators. Furthermore, the increased proliferative activity in the model control group, accompanied by elevated apoptosis, represented a compensatory clearance mechanism of the organism against tumor cells. After estrogen intervention, proliferative activity was significantly inhibited, and this compensatory apoptotic response was correspondingly weakened, which was also an important reason for the downregulation of apoptosis levels.

Pearson correlation analysis showed a strong positive correlation between Ki-67 and PCNA expression (r = 0.92), confirming the consistency of these two markers in detecting intestinal epithelial proliferation. Both markers were significantly correlated with tumor size, indicating that the proliferative activity of intestinal epithelial cells was a core factor influencing tumor growth in colon mucinous adenocarcinoma. A moderate negative correlation was observed between Ki-67 and the TUNEL-positive rate (r = −0.60), further validating the antagonistic relationship between tumor cell proliferation and apoptosis. Meanwhile, ERβ expression in tumor tissues of the model control group was 2.3 times that of the normal control group, and it was further upregulated by 52% after estrogen intervention, while ERα expression showed no significant regular changes. This clearly confirmed that the antitumor effect of estrogen was primarily mediated through the ERβ receptor. It is noteworthy that the highly invasive and highly drug-resistant characteristics of colon mucinous adenocarcinoma are closely related to the stemness of cancer stem cells, and the NF-κB signaling pathway is a key pathway regulating the stemness of cancer stem cells and shaping the malignant phenotype of the disease. Studies have confirmed that activation of the NF-κB signaling pathway is accompanied by downregulation of proliferation markers such as Ki-67 [26], which echoes the results of this study, where estrogen inhibited Ki-67 expression and regulated the tumor microenvironment. It is speculated that estrogen may inhibit the overactivation of the NF-κB signaling pathway through the ERβ receptor, thereby downregulating the stemness characteristics of cancer stem cells, reducing the population of stem cells with tumor-initiating capacity, and ultimately achieving the effect of inhibiting tumor proliferation and invasion. However, the expression of cancer stem cell-like markers and NF-κB downstream factors was not detected in this study, so this regulatory pathway could not be directly confirmed. This represents an

important limitation of this study and warrants further investigation in subsequent experiments.

The animal experimental results of this study provided a novel direction for the clinical treatment of colonic mucinous adenocarcinoma. Estrogen and its receptor ERβ were suggested as potential therapeutic targets for this subtype of colon cancer, and their combined application with existing clinical therapeutic strategies warranted further investigation. It has been previously demonstrated that the combination of 17β-estradiol and anti-PD-L1 antibody inhibited colon tumor growth by reducing PD-L1 expression and increasing the proportion of M1-type macrophages [27], indicating a synergistic effect between estrogen-related signaling pathways and immunotherapy. Meanwhile, in the present study, estrogen was found to ameliorate body weight loss and reduce food intake in the model rats, alleviating tumor-induced cachexia. When combined with metabolic targeted therapy, estrogen might achieve more significant tumor suppression through multi-pathway regulation of the tumor microenvironment. Furthermore, as a special subtype of colon cancer, colonic mucinous adenocarcinoma showed limited response to conventional chemotherapy and targeted therapy. The targeted regulation of this subtype by estrogen might compensate for the deficiencies of current therapeutic approaches, thereby providing experimental evidence for the optimization of clinical treatment strategies.

Nevertheless, several limitations of this study should be acknowledged. First, the experiments were conducted solely using a rat model, and differences in the tumor microenvironment between rats and human colonic mucinous adenocarcinoma exist; therefore, clinical validation using human samples is required before the findings can be translated into clinical practice. Second, although ERβ was confirmed as the primary receptor mediating the effects of estrogen, the upstream and downstream signaling nodes of ERβ were not investigated, and the specific signal transduction pathways through which estrogen regulates tumor growth were not fully elucidated. Third, the expression of cancer stem cell-like markers and NF-κB downstream factors was not analyzed; thus, the association between estrogen and cancer stemness or inflammatory signaling pathways was not directly confirmed, resulting in insufficient depth in the mechanistic investigation. Fourth, estrogen was not administered in graded doses; consequently, the dose–response relationship underlying its tumor-suppressive effects was not clarified, and its regulatory effects at different stages of tumor development were not explored. These limitations represent key directions for future research.

5. Conclusions

This study demonstrated that estrogen significantly inhibited the proliferation of intestinal mucosal epithelial cells by downregulating the expression of proliferation-related factors such as Ki-67 and PCNA, thereby effectively attenuating tumor growth and invasive progression of colonic mucinous adenocarcinoma. Additionally, estrogen ameliorated intestinal tissue structural damage and alleviated tumor-induced cachexia. Estrogen intervention resulted in a mild reduction in the apoptosis level of intestinal epithelial cells in tumor-bearing rats, and its tumor-suppressive effect was primarily mediated through the upregulation of ERβ receptor expression in tumor tissues. It was speculated that estrogen might exert its anti-tumor effects against colonic mucinous adenocarcinoma by modulating immune responses and cell cycle progression within the tumor microenvironment via the ERβ receptor.

This study identified ERβ as a potential therapeutic target for colonic mucinous adenocarcinoma, providing experimental evidence for precision treatment of this colorectal cancer subtype and establishing a foundation for exploring combination regimens integrating estrogen with immunotherapy and metabolic targeted therapy. Nevertheless, limitations of this study included its reliance on an animal model alone, the lack of elucidation of upstream and downstream signaling pathways of ERβ, the absence of investigation into the association with cancer stemness, and the omission of dose–response analysis. Future research should focus on further elucidating the specific signaling pathways mediated by ERβ, detecting cancer stem cell-related markers, conducting dose-gradient experiments, and validating the findings using clinical samples to further clarify the clinical applicability of estrogen in the treatment of colonic mucinous adenocarcinoma.

CRediT authorship contribution statement

Ke Wang: Writing – original draft. **Yumin Yue:** Writing – original draft. **Xiaoli Liu:** Writing – original draft. **Meng Fan:** Writing – original draft.

Ethical approval (animals)

All experimental protocols were approved by the Xi'an Fengcheng Hospital. All methods were carried out in accordance with relevant guidelines and regulations. All methods are reported following ARRIVE guidelines (https://arriveguidelines.org/) for the reporting of animal experiments. All experiments strictly comply with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

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Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

All data generated or analyzed in this study are included in this article. Relevant raw data can be reasonably obtained from the corresponding author upon request.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2026.100725.

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