

Original Article

Role of Wnt/ β -catenin signaling pathway in the repair of intestinal mucosa associated with crypt stem cell in a rat model of abdominal compartment syndrome

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Abstract: The Wnt/ β -catenin signaling plays a vital role in the maintenance of intestinal crypt epithelial cell regeneration, but its role in intestinal epithelial cell after injury is poorly understood. This study investigated the role of Wnt/ β -catenin signaling on the regeneration of intestinal crypt stem cells in Abdominal Compartment Syndrome (ACS) rat model. Arterial blood was collected for biochemical analysis and then microscopic observation showed extensive ileal villus damage after prolonged exposure to Intra-abdominal pressure. Moreover, ACS injury increased the mRNA and protein expressions for Wnt-1, β -catenin, transcription factor 4 (TCF4), and c-Myc. Immunofluorescence staining confirmed the increased proteins of β -catenin and c-Myc in the intestinal crypt stem cells according to morphology. Liver, kidney, heart and other organs were injured after ACS indicated by the blood biochemical analysis, and ACS causes the intestinal mucosal injury, which aggravates with time, and the Wnt/ β -catenin signaling pathway is involved in intestinal epithelium regeneration, which is associated with crypt stem cells in early stage after ACS injury.

Keywords: ACS, Wnt/ β -catenin signaling pathway, intestinal crypt stem cell

Introduction

Abdominal compartment syndrome (ACS) is usually induced by intra-abdominal pressure (IAP) greater than 20 mmHg and results in several organs dysfunctions, failure, or death [1]. ACS is the most common cause of retroperitoneal bleeding, pancreatitis, intestinal obstruction, tension ascites, peritonitis, and severe visceral edema because of fluid resuscitation. The affected systems include cardiovascular, respiratory, kidney, central nervous system, internal organs, and systemic accumulation [2]. The incidence of ACS in intensive care unit is 5%-12% [3], and the mortality rate of ACS is 63%~75% [4]. Therefore, timely and effective diagnosis and treatment of ACS are some of the important problems faced by clinicians. Studying the pathogenesis to elimination ACS is particularly important in achieving a good therapeutic effect [5]. Increased IAP, which usually induces multiple organ dysfunctions in

several systems, can damage the function of the intestinal tract [6]. The intestinal mechanical barrier, which is composed of the mucosal epithelium, lamina propria and muscularis mucosa, as well as the intestinal epithelial cells, including absorptive cells, goblet cells, Pan's cells, stem cells and endocrine cells, are very important in this process. Among these cells, stem cells located in the intestinal crypt region showed a strong capacity to maintain proliferation and differentiation for epithelium repair. Therefore, stem cells play an important role in the intestinal mucosal regeneration and repair [7]. Under the influence of physiological or pathological signals, intestinal stem cells could proliferate and differentiate into absorptive cells, goblet cells, Pan's cells and endocrine cells. However, the underlying mechanism is yet to be determined.

Recent studies showed four main signaling pathways that maintain intestinal stem cells

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renewal and promote cell differentiation [8]. The most important pathway is the Wnt/ β -catenin signaling pathway [9], and Wnt1, β -catenin, TCF4, and c-Myc are key molecules in the signaling pathway. Wnt/ β -catenin signaling pathway is a signal transduction system, which plays an important regulation role in cell proliferation and differentiation. Previous data show that the activation of Wnt/ β -catenin signaling promotes the growth of cancer stem-like cells [10-12]. The current study on the Wnt/ β -catenin signal pathway in gastrointestinal tract mainly focuses on its role in cancer [10, 13, 14]. The Wnt/ β -catenin signaling pathway is involved in the interaction of stem cells and the extracellular matrix in other models [15]. However, the process on how Wnt/ β -catenin signaling pathway promotes the proliferation and differentiation of intestinal crypt stem cells as well as the associated mechanism in the repair of intestinal mechanical barrier damage of ACS is poorly understood. In-depth study of Wnt/ β -Catenin signaling pathway for ACS intestinal stem cell proliferation/differentiation and intestinal mechanical barrier repair as well as further exploration on the protective mechanism are necessary to provide a theoretical basis for the clinical treatment of ACS patients with intestinal mechanical barrier.

Materials and methods

Animals and groups

A total of 48 healthy adult male SD rats weighing 250-300 g were provided by the Experimental Animal Center of Kunming Medical University. The rats were randomly divided into a sham group and an ACS group (IAP at 20 mmHg), with 24 rats in each group. The ACS groups was randomly divided into four subgroups per the IAP duration (0.5, 2, 4, and 6 h), with six rats in each subgroups. The sham and ACS groups were compared.

Simple IAP meter production

A 40 cm long transfusion tube was cut off from the infusion tube. One end was retained with filter and connected with the intravenous infusion needle. The other end was linked with the three-way tap; the connecting pipe was pulled off between the table type sphygmomanometer

and the cuff. The syringe with 50 ml transmitted nitrogen was linked to the infusion tube of the three-way tap.

Preparation of animal model

Before the experiment, the rats were fasting for 12 h and provided with water. The following two groups were created. (1) The ACS group (n=24) was anesthetized by intraperitoneal injection of 3% pentobarbital ($1 \text{ ml} \cdot \text{kg}^{-1}$). The supine was fixed, and the chest, abdomen and right lower extremity skin were prepared by disinfection. Indwelling cannula needle (No. 16) was inserted into the left lower abdomen, and purse-string sutures and ligature-knotted needle were administered to prevent the needle from coming out. The 50 ml syringe was used to inject nitrogen at a rate of $20 \text{ ml}/10 \text{ min}^{-1}$ into the abdominal cavity of the rats by the three-way pipe until the IAP value was increased to 20 mmHg and maintained for 30 s. The three-way pipe was turned to combine the blood pressure with the animals' abdominal pressure. The values of IAP were maintained and monitored until the scheduled time. (2) In the sham group (n=24), the needle was also removed after anesthetization, but the rats were not further injected with nitrogen. Lastly, the samples were harvested at the corresponding time.

Specimen collection

The ACS rats were subjected to IAP (20 mmHg) for 0.5, 2, 4, and 6 h. The rats were then fixed with supine position, and a cut was performed along the central line of skin to expose the chest. After the xiphoid was lifted with tweezers, and the diaphragm was removed, the ribs and muscles alongside the chest were incised to expose the heart. Briefly, 150 ml of physiological saline was perfused into the ascending aorta from the left ventricle, and 2.5 cm of small intestine was removed along a 5 cm area from the ileocecal valve. Mesenteric tissue was removed, washed with saline, and fixed with 4% paraformaldehyde. Subsequently, about 12 cm of small intestinal tissue and the mesentery was removed and cleaned after dividing them into four sections. The parts were placed in a frozen storage tube with RNA enzyme following the order of 1, 2, 3, and 4, and were placed in the refrigerator at -80°C for preservation.

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Blood biochemistry

After the serum was taken out of from -80°C , the levels of bilirubin, aminotransferase, creatinine, urea nitrogen and prothrombin time were detected by automatic biochemical analyzer, and the changes of various indexes and organ function were analyzed.

Hematoxylin eosin (HE)-staining

After the specimens were fixed in 4% paraformaldehyde for 4 h, 15% sucrose-paraformaldehyde was used overnight until the specimens sunk. The tissue was then placed into 30% sucrose mixed with paraformaldehyde until sinking. The tissue was used as frozen medium for the frozen section (Leica, CM1860, Germany) with thickness of 6 μm . HE staining was performed as following: the sections were washed thrice with distilled water (with each wash lasting for 5 min) and then stained by hematoxylin for 5 min. After washing with distilled water for 3 s, 1% hydrochloric acid-ethanol was added for 3 s, and the sections were washed with distilled water for 5 min and stained by 0.5% eosin for 5 s. The sections were slightly washed with distilled water for 2 s, dehydrated using grade ethanol, and cleared with xylene twice (with each wash lasting for 2 min). Lastly, the sections were sealed with neutral gum and were observed under normal optical microscope (Ningbo Sunny Instruments, CX40) and image acquisition.

Quantitative real-time transcription-polymerase chain reaction (QRT-PCR)

After total RNA was extracted using Trizol intestinal tissue kit (Life Technologies) and precipitated with chloroform and isopropanol, the RNA was reverse transcribed into first strand cDNA using the reverse transcription kit (Thermo, #K1622 For 100 rxns). Genes for Wnt-1 (Rn01761722_m1), c-Myc (Rn00561-507_m1), TCF4 (Rn00584481_m1), β -catenin (Ctnnb1) (Rn00584431_g1), and Actb (Rn00-667869_m1) were amplified using mixed reagents (probe and primer) (Applied Biosystems), whereas Actb (β -actin) was used as internal control. Reaction for QRT-PCR was performed by using the required instrument (BIO RAD, CFX96 Touch™ Thermal Cycler). The reaction system is as follows: initial deformation 95°C , one cycle for 5 min; 95°C for 10 s, annealing for

10 s, and 60°C for 20 s for 40 cycles. Lastly relative mRNA expression was calculated by $2^{-\Delta\Delta}$.

Western blot analysis

The nuclei of small intestine (about 200 mg) were extracted by the nuclear extraction kit (Solarbio, Cat#SN0020, CHN), and the nuclear proteins were extracted by protein lysis kit (RIPA). Another sample (100 mg) was used to extract the total protein from the tissue with RIPA. Briefly, 100 μg protein (nuclear protein 40 μg) was separated by sodium dodecyl sulfate polyacrylamide gel, electrophoresis and transferred in the nitrocellulose membrane. The membrane was incubated with primary antibody and secondary antibodies (goat anti-rabbit/goat anti-mouse, 1:5000, Zhongshan Golden Bridge Biotechnology Co., Ltd. China). Known as anti-rabbit β -catenin/CTNNB (1:750, Yunnan Chien Technology, Affinity, DF6794), anti-rabbit TCF4 (1:750, Yunnan Chien Technology, Affinity, DF627), anti-rabbit c-Myc (1:1000, Yunnan Chien Technology, Affinity, AF0358), anti-rabbit Wnt1 (1:1000, Yunnan Chien Technology, Affinity, AF5315c), and anti-mouse β -actin (1:1000, Yunnan Chien Technology, Affinity, BF0198) were used as the internal reference of the total protein. Anti-rabbit LMNB1 (1:1000, GeneTex, LaminB1) is the internal reference of the nuclear protein. Quantitative analysis was performed with Image J (BIO RAD, ChemiDoc™ XRS + Imaging System) software using the preferred gray values.

Immunofluorescence

After rinsing with 0.01 mol/L of PBS, the sections fixed with 4% paraformaldehyde and sucrose gradient were dehydrated for 5 min, and the procedure was repeated thrice. Goat serum (5%) was then added to close non-specific sites at room temperature for 1 h. Anti-rabbit β -catenin/CTNNB (1:100, Yunnan Chien Technology, Affinity, DF6794), anti-rabbit c-Myc (1:100, Yunnan Chien Technology, Affinity, AF0358), and anti-mouse Lgr5 (1:200, Yunnan Chien Technology, Origene, CF503155) were used to incubate the sections in the fridge at 4°C overnight (over 18 h). Next, the sections were rinsed five times with 0.01 mol/L of phosphate-buffered saline with Tween (PBST) with each wash lasting for 5 min. The following steps

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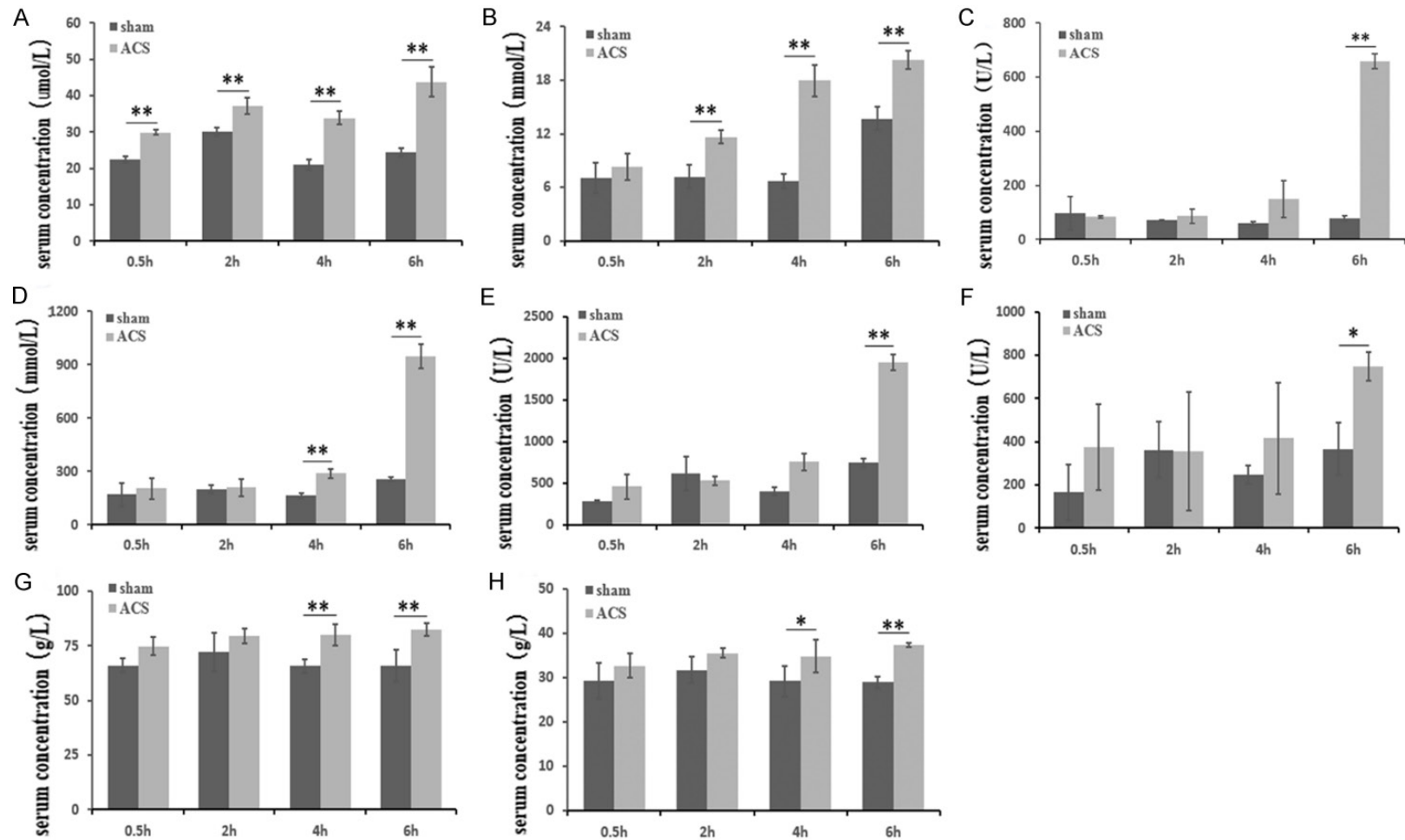


Figure 1. Changes of biochemical indexes in blood of rats after ACS. A. Creatinine (CREA); B. Urea (UREA); C. Alanine aminotransferase (ALT); D. Aspartate amino transfer enzymes (AST); E. Creatine kinase (CK); F. α -hydroxybutyrate dehydrogenase (α -HBDH); G. Total protein (TP); H. Albumin (ALB). Data were presented as means \pm SD (n=6). *P < 0.05 versus sham group. **P < 0.01 versus sham group.

were performed in weak light: the corresponding fluorescent secondary antibody (green, Goat anti-mouse, IgG, Abbkine, Dylight 488, #A23210; green, Goat anti-rabbit, IgG, Abbkine, Dylight 488, #A23220; red, Goat anti-rabbit, IgG, Abbkine, Dylight 594, #A23430) were used to incubate the sections at 37°C for 30 min. The sections were then rinsed with 0.01 mol/L of PBST for 5 min, and the process was repeated thrice. Lastly, 4',6-diamidino-2-phenylindole (DAPI) staining was performed. The stained sections were observed with fluorescence microscope (Leica, DM4000B, Germany), and images were acquired.

Statistical analysis

SPSS19.0 statistical software was used for statistical analysis. The statistics are expressed as mean \pm standard deviation (SD). Single factor analysis of variance was used for comparison among groups. $P < 0.05$ indicates statistically significant difference.

Results

Changes of blood biochemical indexes in rats after ACS

After the ACS processing with the change of time, compared with the corresponding sham group, the urea (UREA), total protein (TP), albumin (ALB), creatinine (CREA), aspartate amino transfer enzymes (AST), alanine aminotransferase (ALT), creatine kinase (CK) and α -hydroxybutyrate dehydrogenase (α -HBDH) levels increased significantly, and it is the most obvious at 6 h. P values were 0.001, 0.001, 0.002, 0.001, 0.001, 0.001, 0.002 and 0.005, respectively, all of which were less than 0.05, and there were significant differences. Some blood biochemical parameters are shown in **Figure 1**.

ACS causes intestinal mucosal damage and differences in Wnt/ β -catenin signaling molecules expression in the ileum tissue

Obvious damage to the intestinal mucosa of the sham group was not observed under the light microscope, which showed the normal columnar epithelium (**Figure 2Aa**). The ACS group, in the presence of IA hypertension (IAH), showed different degrees of edema from epithelial structure transformation with shedding and atrophy of the villi. The extent of damage

was positively correlated with the duration of IAH maintenance, especially at 4 and 6 h ACS subgroups (**Figure 2Ad** and **2Ae**).

QRT-PCR was used to detect the expressions of Wnt-1, β -catenin, TCF4, and c-Myc mRNA in the ileum tissue samples. mRNA levels for each factor at 0.5, 2, 4, and 6 h in sham and ACS groups are shown in **Figure 2B-E**. Each gene showed initial change, which then reduced with time. Wnt1, β -catenin, and TCF4 were upregulated most obviously at 2 h (** $P < 0.01$) (**Figures 2B, 1D**), whereas c-Myc expression was highest at 4 h (**Figure 2E**).

Western blot verified that Wnt-1, β -catenin, TCF4, and c-Myc protein expressions (**Figure 2F-J**) and nuclear β -catenin protein expression (**Figure 2K** and **2M**) are consistent with the results of QRT-PCR, showing that the relative protein expression increased and then gradually decreased over time. Moreover, the expressions of Wnt-1, β -catenin, and nuclear β -catenin protein in sham group were higher than in the ACS group with prolonged period of observation (4 and 6 h) (** $P < 0.01$) (**Figure 2G, 2H** and **2M**). Compared with the sham group, the expression of each factor in the ACS group was downregulated with increasing time.

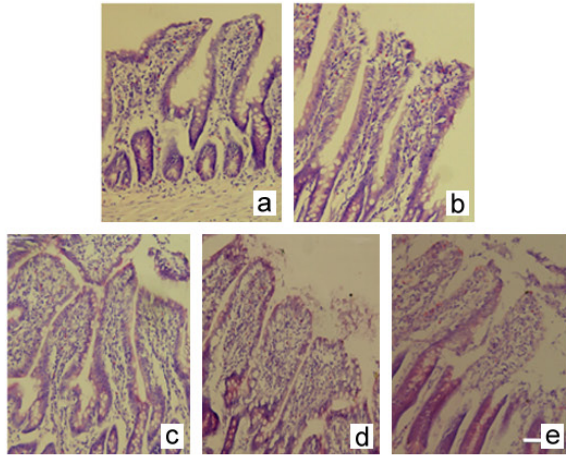
Localization of β -catenin and c-Myc in the small intestinal crypt and the expression of β -catenin and c-Myc after ACS by immunofluorescence

Localization of Wnt/ β -catenin pathway key proteins, including β -catenin and c-Myc, was observed in the small intestinal crypt after ACS, as detected by immunofluorescence. β -catenin and Lgr5 (**Figure 3A**) as well as c-Myc and Lgr5 (**Figure 3B**) were respectively double labeled. β -catenin and c-Myc coincided with the stem cell marker Lgr5 in small intestinal crypt.

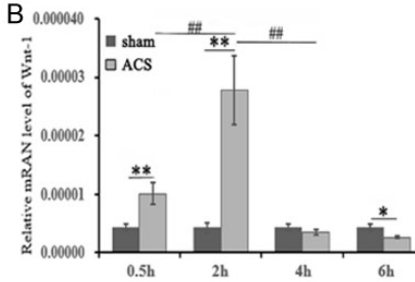
Compared with the sham group, the expression of β -catenin in the nucleus initially increased and then decreased. In detail, ACS group reached an expression which is 2.6 times (** $P < 0.01$) than that of the sham group, with the highest expression of β -catenin being observed at 2 h after injury. However, result was not statistically significant between the ACS group and sham group when the time of IAH extended to 6 h (**Figure 3E**). c-Myc also showed a similar trend with the β -catenin. With prolonged IAH period,

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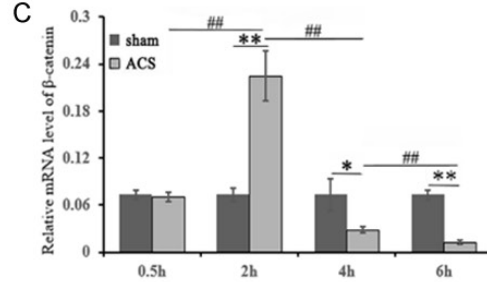
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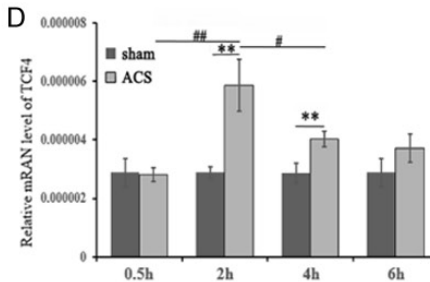
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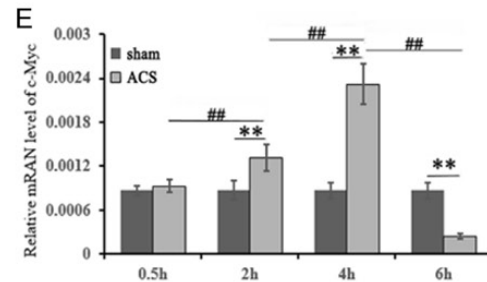
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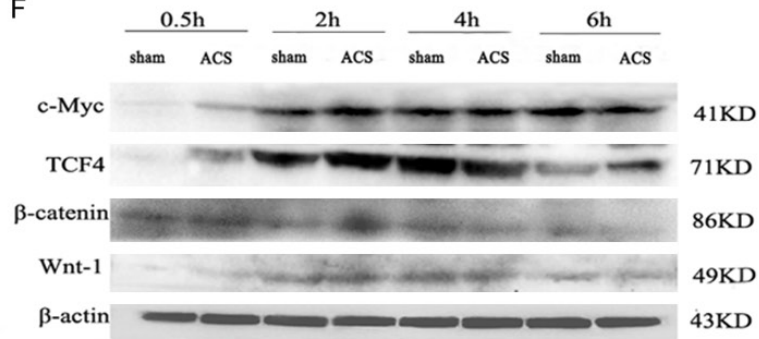
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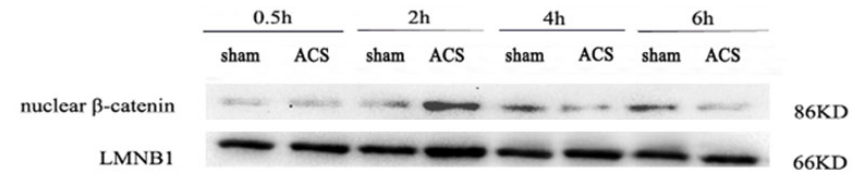
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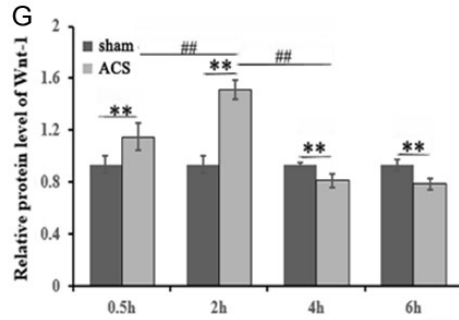
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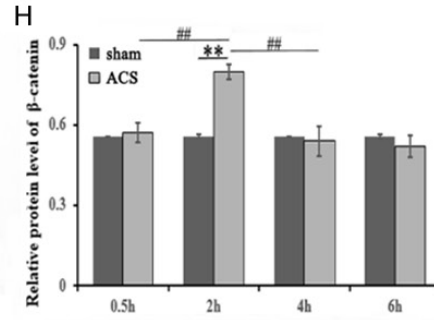
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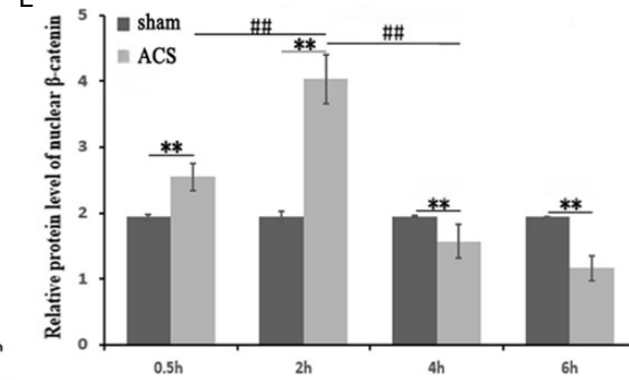
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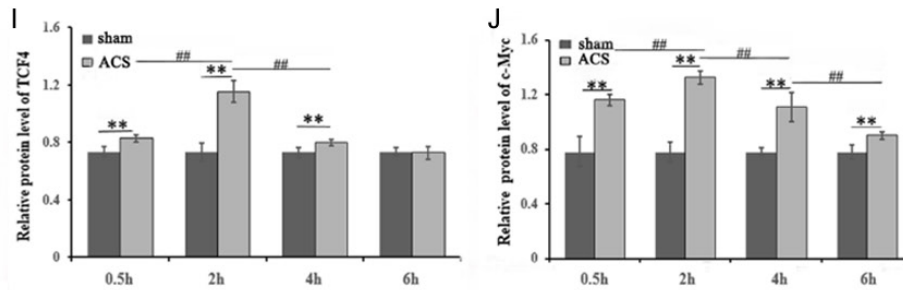


Figure 2. ACS causes intestinal mucosal damage and differences in Wnt/ β -catenin signaling molecules expression in the ileum tissue. Observation on the pathological changes of ileum mucosa was conducted by light microscope (A). (Aa) represents the sham group; (Ab-e) represent 0.5, 2, 4, and 6 h ACS group. $N \geq 3$ for (A); scale bar, (A) 100 μ m. (B-E) Representative mRNA expression of Wnt-1 (B), β -catenin (C), TCF-4 (D), and c-Myc (E) in the ileum mucosa of ACS group compared with the sham group at different time points (0.5, 2, 4, and 6 h), as determined by QRT-PCR. The expression of each target gene was normalized to β -actin. (F) Protein expressions of Wnt-1, β -catenin, TCF4 and c-Myc in the ileum mucosa of ACS group compared with the sham group at different time points (0.5, 2, 4, and 6 h), as measured by Western blot. β -actin was used as a loading control. (K) The nucleus of the ileum mucosa was extracted to detect the change in the nuclear β -catenin protein in ACS group compared with the sham group, as measured by Western blot. LMNB1 was used as loading control. (G-J, L) Quantification of band density was performed with Image Lab software. Data were presented as mean \pm SD. $n=6$ for (B-E); $n=3$ for (G-J, L). * $P < 0.05$ versus sham group. ** $P < 0.01$ versus sham group. # $P < 0.05$ versus comparison among the ACS groups. ## $P < 0.01$ versus comparison among the ACS groups.

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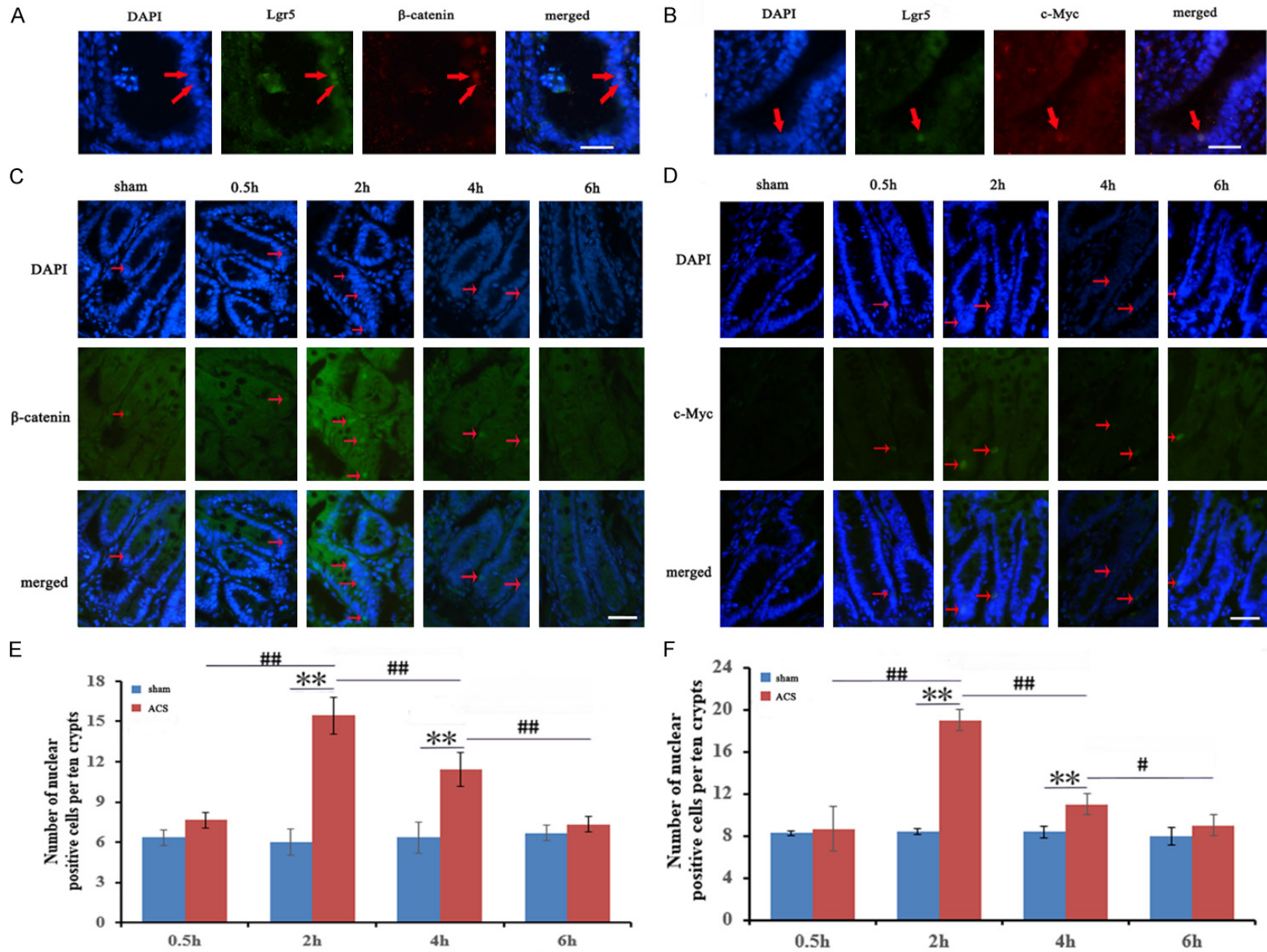


Figure 3. Localization of β -catenin and c-Myc in small intestinal crypt and the expression of β -catenin and c-Myc after ACS by immunofluorescence. (A) Immunofluorescence staining of Lgr5 (green) and β -catenin (red). (B) Immunofluorescence staining of Lgr5 (green) and c-Myc (red). The samples (A and B) were derived from the ileum after ACS for 2 h. (C and D) The effect of ACS on the expressions of β -catenin (green) and c-Myc (green) in the intestinal crypt in the ileum mucosa of ACS group compared with that of the sham group at different time points (0.5, 2, 4, 6 h). Blue colour (A-D) represents DAPI stain. $N \geq 3$ for (A-D); scale bar, (A-D) 50 μ m. Data were presented as mean \pm SD. $n=3$ for (E and F). * $P < 0.05$ versus sham group. ** $P < 0.01$ versus sham group. # $P < 0.05$ versus comparison among the ACS groups. ## $P < 0.01$ versus comparison among the ACS groups.

the protein expressions initially increased and then decreased, but statistical significance was not observed when results were compared with those for the sham group at 6 h (**Figure 3F**).

Discussion

ACS is often accompanied by new organ dysfunction or failure [1]. The affected systems include cardiovascular, respiratory, kidney, central nervous system, internal organs, and finally accumulated systemic [2]. Our biochemical results showed that, compared with the sham group, the ALT and AST were significantly increased, suggesting a detected liver deterioration. Compared with the sham group, the changes of CREA and UREA in the early appeared to indicate that the kidney may be the organ that was affected by ACS earlier. Changes of the CK and α -HBDH may suggest that cardiac function was also affected. Elvevoll B et al. used a pig model of IAP proved that elevation of intra-abdominal pressure has an immediate impact on microvascular fluid extravasation leading to plasma volume contraction, reduced cardiac output and deranged perfusion of abdominal organs [16]. Our experiments indicated that elevated ALB in the blood vessels leads to fluid extravasation of plasma volume contraction, consistent with previous reports. At the same time, we found that the TP in both 4 h and 6 h was increased, while the changes of the TP may provide new biomarkers for the ACS.

Previous experiments showed that ACS leads to intestinal perfusion deficiency, ischemia, intestinal mucosal acidosis, capillary leakage, intestinal edema, increased permeability, and bacterial translocation [16-19]. Ischemia and hypoxia caused by intestinal hypoperfusion accelerate the intestinal mucosa damage. Our experiments confirmed that the structure of small intestinal villi was damaged under ischemia and hypoxia conditions, indicating that the intestinal epithelial cell loss and edema of

the intestinal wall. These morphological changes in the structure through capillary leak and bacterial translocation may promote the progression of ACS disease [20].

The Wnt/ β -catenin signaling pathway plays a critical role in the maintenance of proliferating crypt epithelial cells [9]. Loss of Wnt/ β -catenin signaling pathway inhibits the proliferation of intestinal crypt cells and ultimately causes the destruction of epithelium [21, 22]. Maintenance of adult crypt proliferation is dependent on Wnt, as indicated by the transgenic expression of Wnt receptor antagonist Dkk1 and conditional deletion of β -catenin or TCF4 in adult mice [22-24]. In this study, we discovered that ACS causes a transient rapid proliferation of intestinal crypt epithelial cells in early period of the disease. Changes in the mRNA and protein expressions of Wnt-1, β -catenin, TCF-4, and c-Myc on mRNA and protein levels significantly increased in the intestinal crypt epithelial cells early after ACS but were gradually inhibited over time.

According to our knowledge, this study is the first time to demonstrate the upregulation of β -catenin expression in the early phase after ACS intestinal injury. In the small intestinal mucosa, β -catenin was expressed on the spindle axis of the villi. However, the β -catenin is located in the cell membrane of the chorionic villi cell, whereas in the lower one-third of the crypt where the stem cells are located, β -catenin is mainly located in the nucleus. Nuclear β -catenin, the hallmark of Wnt signaling, is observed throughout the crypts of the intestine [25], suggesting its role on the regulation of crypt epithelial cell proliferation. At present, the general use of total β -catenin and p β -catenin is to detect Wnt/ β -catenin activity [26, 27], but nuclear β -catenin activates the Wnt/ β -catenin pathway. Our experiments examined the changes in the expression of β -catenin in the nucleus by extracting the nuclear proteins from the ileum after ACS. In

this study, we observed that β -catenin expression increased in the intestine at 2 h after ACS. Corresponding with the increased β -catenin expression, the expression of Wnt-1, TCF4 and c-Myc on proteins were also significantly upregulated at 2 h after ACS. Immunofluorescence examination of the intestinal tissues was performed to further confirm the activation of Wnt/ β -catenin signaling early after ACS. Previously, 16 stem cells were estimated to be present in the intestinal crypt. However, when considering the number of actual functional stem cells, this number was reduced to 5-7 per intestinal crypt [28, 29]. Localization of intestinal stem cells has many biological markers, of which Lgr5 is one of the most common [9]. This experiment proved that β -catenin is expressed in the small intestine stem cells by double labeling with Lgr5. T. Dorfman et al. [30] established the model of diabetic rats and confirmed the change in the intestinal crypt cells containing the nuclear β -catenin with immunohistochemical staining. We also used a similar method to count the number of nuclear positive cells per ten crypts of the same samples. Results showed that Wnt/ β -catenin signaling components was expressed in the intestinal crypts, and that the number of cells expressing Wnt/ β -catenin signaling components were significantly increased immediately after ACS. Results showed that Wnt/ β -catenin signaling was activated in the intestine immediately after ACS and was involved in the proliferation of crypt epithelial cells after ACS.

Our results suggest that the Wnt/ β -catenin signaling pathway is involved in the activation of intestinal crypt epithelial cells. In addition to the Wnt/ β -catenin signaling pathway, BMP, Notch, and EGF pathways also play important roles in the homeostasis of the intestinal epithelium and in the establishment of the Crypt-Villus Axis [8]. The BMP signal affects the proliferation and self-renewal of small intestinal stem cells by directly inhibiting the Wnt signal [31]. Notch1 may be downstream of Wnt/ β -catenin signaling pathway [32].

Conclusion

The liver, kidney, heart and other organs were injured after ACS indicated by the blood biochemical analysis, and the Wnt/ β -catenin signaling pathway is involved in the regeneration

of the intestinal epithelium early after ACS and in the activation of crypt stem cells of intestine but is gradually inhibited over time. However, the precise molecular mechanisms in ACS within the intestinal epithelium should be further clarified.

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Disclosure of conflict of interest

None.

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