Rosmarinic acid protects against ulcerative colitis by regulating macrophage polarization depending on heme oxygenase-1 in mice

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Abstract
Ulcerative colitis (UC) is an unknown-cause inflammatory disease of colorectum. At present, there are no specific therapeutic drugs. We found that rosmarinic acid (RA) can significantly improve UC and further explored the relevant cellular and molecular mechanisms. Firstly, using F4/80 as marker for mouse macrophages, we found there were large numbers of macrophages infiltrating into colonic tissue of dextran sulfate sodium (DSS)-induced mice UC model. Meanwhile, RA markedly improved weight loss, diarrhea, hematochezia and colonic inflammation in mice with DSS treatment. Further, RA changed macrophage polarization in mouse colon, showing that classical activation (M1) phenotype decreased, alternative activation (M2) phenotype increased, and M1/M2 ratio reversed by Real-time PCR. In vitro, we cultured the peripheral blood macrophages (PBM) and found that RA inhibited PBM M1 polarization and favored M2 polarization directly. Heme oxygenase-1 (HO-1) mediated the anti-inflammatory effect of RA. RA induced HO-1 expression in PBM, and the HO-1 inhibitor, zinc protoporphyrin, blunted the inhibitory effect of RA on lipopolysaccharide (LPS)-induced nuclear factor-kappa B (NF-κB) translocation and M1 polarization. In addition, blocking NF-κB signal has no effect on the role of RA. In conclusion, RA protects against UC by regulating macrophage polarization depending on HO-1. These data suggest that reversing macrophage polarization can be used as a strategy for UC treatment and RA is an effective drug to cure UC by regulating macrophage polarization.

Keywords
heme oxygenase (HO)-1, inflammation, macrophage polarization, rosmarinic acid, ulcerative colitis

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Introduction
Ulcerative colitis (UC) is an unknown-cause inflammatory disease of colorectum, which is characterized by chronicity and relapse. The main clinical manifestations are abdominal pain, diarrhea, and purulent stool. Toxic megacolon, intestinal perforation, hemorrhage, and canceration occur in severe cases. Despite having some drugs, there are still a large quantity of patients with poor therapeutic effect.1,2 Numerous immune cells and inflammatory factors are involved in the development of ulcerative colitis. Studies have verified that T-cells play

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crucial roles in driving intestinal inflammation.3,4 However, many therapeutic ways aiming at specific T-cell have largely been defeated.5 Therefore, it is necessary to research deeply on other immune cells for completely understanding pathological mechanism and improving therapeutic strategies of ulcerative colitis.

Macrophages are significant immune cells belonging to mononuclear phagocyte system, which ubiquitously populate in almost all organs and tissues and exhibit great functional diversity in development, homeostasis, and disease. Through the migration, phagocytosis and secretion, macrophages have critical roles in inflammation.6,7 But in UC, the role of macrophages is not clear. Macrophage polarization is a hotspot in immunology research at present. In response to the cytokines interferon gamma (IFNγ) and activation of toll-like receptors (TLRs) by lipopolysaccharide (LPS) or interleukin (IL)-4/IL-13 signaling, macrophages undergo M1 (classical) or M2 (alternative) activation. M1 macrophages mainly secrete some proinflammatory cytokines (such as tumor necrosis factor-alpha (TNF-α), IL-1, and IL-6) to enlarge inflammation and sometimes exacerbate the tissue damage in overactivity. In contrast, M2 phenotype mainly express anti-inflammatory cytokines (such as tumor necrosis factor-alpha (TNF-α), IL-1, and IL-6) to enlarge inflammation and sometimes exacerbate the tissue damage in overactivity. In contrast, M2 phenotype mainly express anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGFβ), that are characterized by its capacities of inhibition of inflammation and tissue remodeling.8,9 Macrophage polarization toward different ways has vital function in the evolution of inflammatory diseases.10,11

Rosmarinic acid (RA, an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid) is widely found in the plant kingdom, such as Rosemary, Prunella vulgaris, and Oregano.12,13 Studies have indicated that RA possesses comprehensive biological effects of anti-oxidant, anti-inflammatory, and anti-ischemia.14,15 However, whether RA can treat UC remains unclear and few studies have focused on the relationship between RA and macrophages. In this study, we found there were large numbers of infiltrating macrophages in colonic tissue of DSS-induced mice UC model and investigated the molecular mechanism of RA improving UC through regulating macrophage polarization.

**Materials and methods**

**Materials**

Rosmarinic acid (RA) was from Sigma-Aldrich (R4033, St. Louis, MO). GlutaMAX Dulbecco’s modified Eagle’s medium (DMEM) and RPMI 1640 was from HyClone/Thermo Scientific (Scoresby, VIC, Australia). Fetal bovine serum (FBS) was from Ausbian (Australia). Polymerase chain reaction (PCR) reagents were from Applied Biosystems (Foster City, CA). Dextran sulfate sodium (DSS), Lipopolysaccharide (LPS), DAPI, zinc protoporphyrin (ZnPP), BAY 11-7082, and other common reagents were from Sigma-Aldrich (St. Louis, MO).

**Mouse models of ulcerative colitis**

Three percent (wt/vol) DSS was used as drinking water to feed adult male ICR mice (10 in each group) for 9 days, while normal drinking water was used as control. Rosmarinic acid was injected intraperitoneally once a day (10 mg/kg). Disease activity index (DAI) was evaluated by the following parameters: two times a day, (a) diarrhea (0 points = normal, 2 points = loose stool, 4 points = water stool); (b) stool blood (0 points = no bleeding, 2 points = mild bleeding, 4 points = massive bleeding). Mice were sacrificed at the 9th day and colon tissue was fixed with 4% paraformaldehyde.

**Hematoxylin-Eosin (H&E) staining and image analysis of colonic inflammation**

The mice colon tissue specimens were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. For histopathological analysis, paraffin sections (4 μm) were stained with hematoxylin and eosin. Observed and photographed under light microscope, measured the thickness of intestinal muscle layer, and analyzed the degree of inflammation. Inflammation score has two parts: (a) epithelial structure (1 points = prolonged epithelial cell or crypt, 2 points = destruction of barrier, 3 points = ulcer 30% < loss < 60%, 4 points = ulcer > 60%); (b) immune cell (1 points = mild infiltration, 2 points = moderate infiltration, 3 points = severe infiltration).

**Immunohistochemical staining**

Paraffin intestinal tissue samples were sectioned to 4 μm thickness. Anti-F4/80 rat monoclonal antibody (1:100, Santa Cruz Biotechnology, CA) was as primary antibody for mouse samples and immunohistochemical staining was performed with Ultra SensitiveTMSP kit (KIT-9730), DAB Color kit (DAB-1031), citric acid tissue antigen repair solution (MVS-0101), all from Maixin Biotechnology (Fujian, China).
Preparation of L929 conditioned medium

4.7 × 10^5 L929 cells (from the central laboratory of Gansu Provincial Hospital) were inoculated in 75 cm^2 culture flask, and 55 mL complete medium (containing GlutaMAX DMEM + 10% FBS) was added, then were cultured in an incubator for 7 days. Lastly, the supernatant was collected and stored at −20°C after being filtered with 0.45 μm filter membrane.

Peripheral blood macrophages acquisition

Murine (ICR) peripheral blood with heparin treatment was subjected to density gradient (Histopaque-1077) centrifugation at 2000 rpm for 20 min. The mononuclear cells were collected from the interface after centrifugation, washed twice with PBS, lastly cultured for 7 days in the presence of 10% L929-conditioned 1640 medium (replacing culture medium at the third, fifth day). The identification of peripheral blood macrophages (PBM) is assessed using immunocytochemistry analysis of F4/80 expression as described previously. All animal work was performed under the ethical guidelines of the Ethics Committee of Gansu Traditional Chinese Medicine University.

Immunofluorescence staining

PBM were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton X-100 for 15 min. After blocked with 3% BSA, they were incubated with anti-F4/80 rat monoclonal antibody (1:100, Santa Cruz Biotechnology, CA). Cy3-labeled goat-anti-rat IgG (1:100, KPL, MD) was as secondary antibody. At last, nuclei were stained with DAPI. PBM that had been cultivated for 7 days were serum-starved and treated with alone 10 µM of RA or vehicle or mixture of RA and 10 µM of ZnPP for an 12-h period. After that, cells were incubated with anti-nuclear factor-kappa B (NF-κB) p65 rabbit polyclonal antibodies (3 μg/mL; Abcam, Cambridge, UK). Cy3-labeled goat-anti-rabbit IgG (1:100, KPL, MD) was as secondary antibody. Nuclei were stained with DAPI. Cytoplasmic or nuclear fluorescence intensity was analyzed by Cellomics Cell Health Profiling BioApplication software.

Real-time PCR

PBM was pretreated with vehicle or 10 µM of RA for 12 h and further incubated with 1 ng/mL of LPS or with 5 ng/mL of IL-4 both for 6 h. Total RNA was extracted from colon tissue or cultured PBM with or without treatments using an RNeasy kit (Qiagen, Hilden, Germany). Primers used for real-time reverse transcription-polymerase chain reaction (RT-PCR) were as follows: 18S rRNA: sense, 5′-GTAACCCCGTGAAACCCATT-3′; antisense, 5′-CCATCCAAATCGGTAGTTCG-3′. Mouse TNF-α: sense, 5′-GCGAGGTCTGTCCTTCACTA-3′; antisense, 5′-CTGCTGACTGTTGCTTATTTCTG-3′. Mouse IL-1β: sense, 5′-CTCAGCGGTCCGGAGTGC-3′. Mouse NOS: sense, 5′-GGATAAAGCAGGGACATTAAGAA-3′; antisense, 5′-TCGCGCTGGCTCAATAATTTG-3′. Mouse IL-12: sense, 5′-GAGCAATGCCTGACCTCAGA-3′; antisense, 5′-GACCAAATT-3′. Mouse CCL4: sense, 5′-GCTGGTGGACTGCTTCATT-3′; antisense, 5′-GTGCTCAGGCTTCTTGCA-3′. Mouse CD16: sense, 5′-GGAGAATATCGGTGTCAAATGGA-3′; antisense, 5′-CCAGTCAGAAATCACTCCCAGA-3′. Mouse Arg1: sense, 5′-CGTCTGAGGAGTGGAGGTCAGA-3′; antisense, 5′-GAGAATATCGGTGTCAAATGGA-3′. Mouse Mrc2: sense, 5′-GGAGAATATCGGTGTCAAATGGA-3′; antisense, 5′-CCAGTCAGAAATCACTCCCAGA-3′. Mouse Mgl1: sense, 5′-GGAGAATATCGGTGTCAAATGGA-3′; antisense, 5′-CCAGTCAGAAATCACTCCCAGA-3′. Mouse CD206: sense, 5′-GGAGAATATCGGTGTCAAATGGA-3′; antisense, 5′-CCAGTCAGAAATCACTCCCAGA-3′. Mouse Dectin-1: sense, 5′-GGAGAATATCGGTGTCAAATGGA-3′; antisense, 5′-CCAGTCAGAAATCACTCCCAGA-3′. Western blot analysis

Western blot analysis was performed with 50 mg of protein extract. Anti-HO-1 goat polyclonal antibody (1:100, Santa Cruz Biotechnology, CA) was as primary antibody and horseradish peroxidase (HRP)-conjugated antibody (Jackson ImmunoResearch Laboratories, PA, USA) was as secondary antibody. The specific bands were detected using a chemiluminescence reagent (Beijing CoWin Bioscience, Beijing, China). Protein ladders were purchased from Thermal scientific (Thermo Fisher Scientific, MA, USA). β-actin was used as reference.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by ANOVA. p < 0.05 was considered significant.
Results

A large number of macrophages infiltrate colonic tissue of DSS-induced mouse UC model

Inflammatory cell infiltration and gland destruction are the main pathological features of ulcerative colitis. In previous studies, the most concerned inflammatory cells were mainly leukocytes and lymphocytes. However, using F4/80 as marker for mouse macrophages, immunohistochemical staining revealed that a huge amount of macrophages infiltrated into colonic tissue of DSS-induced mouse UC model (Figure 1(a)). This observation prompted that macrophages may play an important role in UC and can be used as target for UC treatment.

RA markedly attenuates the inflammation of colonic tissue of the DSS-induced mouse UC model

In order to evaluate the therapeutic effect of RA on ulcerative colitis, a mouse model of UC was established by orally administered DSS. Using weight changes and DAI to assess the overall situation of mice, we found that RA significantly improved the weight loss of mice at 5 to 9 days (Figure 1(b)), and diarrhea and hematochezia also began to improve obviously on the 3rd day, lasting to the 8th day (Figure 1(c)). Further, HE staining of colonic tissues were performed and colonic inflammation was assessed with thickness of muscle layer and inflammation score. Images and data showed in the
DSS group, the thickness of muscle layer and inflammation score were significantly higher than those in the control group (Figure 2(a) and (b)), and RA displayed an apparent reversal of DSS damage. RA treatment group had a thinner muscle layer, less inflammatory cells infiltration and more complete mucosal structure (Figure 2(c)).

**RA alters the polarization of macrophages in DSS-reduced mouse colonic injury**

In order to further study the cellular mechanism of RA improving UC, polarized markers (M1: TNF-α, IL-1β, nitric oxide synthase 2 (NOS2), ligand 4 (chemokine (C-C motif) (CCL4)), IL-12, and CD16/32; M2: arginase 1 (Arg1), mannose receptor C type 2 (Mrc2), macrophage galactose-type C-type lectin 1 (Mgl1) and CD206) were measured by Real-time PCR. In only RA groups, M1 and M2 markers did not change much compared with the control group. In DSS group, the markers of M1 and M2 both increased but M1 more obviously, representing that M1 macrophages were dominant in intestinal injury. Moreover, except for CD16/32, that RA administration reduced M1 most markers, and elevated M2 markers Mrc2 and CD206 indicate RA reverses M1/M2 ratio in DSS-reduced intestinal injury (Figure 2(d) and (e)). Considering the anti-inflammatory and tissue repair function of M2 macrophages, these results suggest that RA improves UC by regulating macrophage polarity.

**RA inhibits M1 polarization and favors M2 polarization in vitro**

For verifying whether RA directly regulates macrophage polarization, we cultured primary peripheral blood macrophages PBMs (F4/80+ as marker of macrophage) in vitro (Figure 3(a)). Treatment with different irritant, macrophages will polarize to different directions.9 After treatment with LPS or IFNγ, macrophages polarized to M1 phenotype. Furthermore, after stimulation with IL-4, macrophages polarized to M2 phenotype. To detect the changes of the polarized marks in PBMs by real time RT-PCR, we found that the mRNA levels of M1 phenotype markers (TNF-α, IL-1β, NOS2, CCL4, and IL-12) didn’t be changed by RA in non-polarized PBMs, however, their levels significantly decreased for more than or nearly half in M1 phenotype PBMs polarized by LPS with the pretreatment of RA (Figure 3(b)). In addition, RA increased the levels of M2 macrophages markers (Arg1, Mrc2, Dectin-1) in non-polarized PBMs, furthermore, RA enhanced the polarized effect of IL-4 for M2 macrophages through elevate the levels of Arg1, Mrc2, Mgl1, and Dectin-1 (Figure 3(c)). These results suggested RA directly restrains M1 macrophages and promotes macrophages into M2 phenotype.

**RA restricts macrophage polarization toward M1 depending on the inhibition of NF-κB p65 nuclear translocation by increasing HO-1**

NF-κB p65 translocation into the nucleus is the center role in LPS-induced macrophages M1 polarization. Heme oxygenase (HO)-1 has been shown to inhibit NF-κB p65 nuclear translocation.18 Using RT-PCR and Western blot methods, we found that RA significantly elevated the number of HO-1 in LPS-induced M1 macrophages, which increased by 2.5 times at the level of mRNA and more than three times at the level of protein (Figure 4(a) and (b)). In the absence of LPS stimulation, RA still showed similar performance in promoting HO-1. Zinc protoporphyrin (ZnPP) is a specific inhibitor of HO-1 activity. Immunofluorescence images displayed that RA prevented LPS-triggered NF-κB p65 nuclear translocation, and ZnPP reversed the effect of RA (Figure 4(c)). This result was supported by quantitative fluorescence analysis (Figure 4(d)). To further evaluate whether RA regulated macrophages polarization through the NF-κB signaling pathway during LPS-induced M1 polarization, the NF-κB inhibitor BAY11-7082 was used to block the signaling pathway. The mRNA results revealed that the downregulation of M1 markers (TNF-α, IL-1β, NOS2, CCL4, and IL-12) by RA showed few difference in LPS-induced M1 macrophages after the NF-κB signaling pathway was blocked (Figure 4(e) displays the results of TNF-α as a representative). The above results suggest that RA restricts M1 polarization depending on the inhibition of NF-κB p65 nuclear translocation by HO-1 (Figure 4(e)).

**Discussion**

The anti-inflammatory potential of RA has been identified in local and systemic inflammation in the animals.19 However, the role of RA in UC is not clear. In this study, we first systematically studied the role of RA in mice models with ulcerative colitis,
Figure 2. RA improves the inflammation of colonic tissue of the DSS-induced mouse UC model and alters the polarization of macrophages in vivo. UC mice were administered with RA. (a) Representative colonic tissue images of H&E staining were showed and the thickness of muscle layer was measured. (b, c) Statistical results of thickness of muscle layer and inflammation score were showed in different groups. Marks representing macrophage polarization in intestinal tissue were measured by real-time RT-PCR. (d) Classical activation (M1) gene expression. (e) Alternative activation (M2) gene expression. Scale bars: 200μm (a) and 10μm [(a) magnified sections]. *p < 0.05 compared with the control, #p < 0.05 compared with alone DSS group without RA (n = 10/group).
founding that RA improved UC. In the mechanism, the reversal of macrophage polarization by RA has been found to play a key role. Furthermore, that RA-induced HO-1 augment inhibited NF-κB p65 nuclear translocation is crucial in the restriction of macrophage polarization toward M1.

Macrophages display a critical role in innate and adaptive immune reaction. The intestine contains a large number of macrophages, which account for the highest proportion of intestinal immune cells. In the process of intestinal microbial invasion and intestinal epithelial renewal, such a large number

Figure 3. RA inhibited M1 polarization and promotes M2 polarization in vitro. Peripheral blood macrophages (PBM) were cultured in vitro and were exposed to RA for 12 h and further incubated with LPS for M1 polarization or IL-4 for M2 both for 6 h. Marks representing macrophage polarization were measured by real-time RT-PCR. (a) Representative images of immunofluorescence staining for F4/80 (red) were shown in PBM. (b) M1 gene expression. (c) M2 gene expression. Scale bars: 25 µm. *p < 0.05 compared with control, #p < 0.05 compared with alone LPS or IL-4-treated PBM (n = 6).
Figure 4. RA regulates macrophages polarization depending on the inhibition of NF-κB p65 nuclear translocation by increasing Heme oxygenase (HO)-1. PBM were cultured in vitro. (a) The mRNA levels of HO-1 in PBM. (b) The protein levels of HO-1 in PBM. (c) Nuclear translocations of NF-κB p65 were showed by immunofluorescence (red). DAPI was used to display nuclei (blue). Zinc protoporphyrin (ZnPP) is a specific inhibitor of HO-1 activity. (d) The mean fluorescence intensity was measured by high content analysis. (e) M1 gene expression (represented by TNFα). BAY 11-7082 is the NF-κB inhibitor. Scale bars: 25 µm. #p < 0.05 compared with alone LPS without RA (n=6).
of macrophages maintain intestinal mucosal homeostasis through secretion and phagocytosis, and are constantly replaced and renewed by bone marrow-derived mononuclear macrophages (BMM).20 Resident macrophages have strong phagocytosis but weak secretion of inflammatory factors, which play an important role in intestinal homeostasis. The infiltrating intestinal macrophages from the blood circulation have a stronger ability to secrete pro-inflammatory factors and favor inflammatory response.21 In mice colitis, Ly6C\(^{hi}\) monocytes that infiltrated gut differentiate into iNOS-expressing CD64\(^{+}\) inflammatory macrophages.22 In this study, using F4/80 to identify macrophages of mice, we found that there were substantial macrophages in the intestinal specimens of mice, suggesting that macrophages can be used as therapeutic targets.

Rosmarinus officinalis, also known as rosemary, containing abundant rosmarinic acid is used traditionally as an effective remedy for pain and inflammatory diseases. In animal experiment, RA obviously decreased the amounts of spinal proinflammatory markers (cyclooxygenase 2 (COX2), PGE-2, IL-1\(\beta\), matrix metalloproteinase 2 (MMP2), and NO) on both days 7 and 14 in rats that underwent chronic constriction injury, indicating a potential treatment for neuropathic pain.23 In live fibrosis model, RA also exhibited strong inhibitions for the activation of hepatic stellate cells and progression of liver fibrosis through suppressing peroxisome proliferator-activated receptor (PPAR) \(\gamma\).24 However, there are few reports focusing on the relation between RA and the polarization of macrophages. Macrophages are divided into binary classifications that refer to distinct programs of activation, known as classic activated M1 that defined by responses to LPS or IFN\(\gamma\), and alternatively activated M2 by IL-4/IL-13.11 Although the two forms cannot represent the complex in vivo setting where multitudinous inflammatory factors interact to define the eventual differentiated status of macrophages, it is widely believed that M1 macrophages play a proinflammatory role in a variety of acute and chronic inflammatory diseases, while M2 are involved in the resolution of inflammation and the remodeling of tissue. In this study, we proved that M1 macrophages were dominant in UC. In vitro, through culturing primary peripheral blood macrophages, we found that RA remarkably restrained M1 macrophages and facilitated macrophages into M2 phenotype. In view of the large intestinal macrophage infiltration in ulcerative colitis and the role of RA in regulating macrophage polarization, RA may be used to treat ulcerative colitis. In our experiments, this hypothesis was confirmed that RA significantly improved UC and changed the polarization of macrophages in UC mice. The limitation of this study is that the sample size of mice in each group is not large enough (10 per group). In order to confirm the conclusion better, it may be needed to expand the sample size or to use different kinds of animals.

A predominance of NF-\(\kappa\)B p65 signaling pathway activation induces macrophages M1 polarization, resulting in proinflammatory functions.17 In this study, stimulating macrophages with LPS can make NF-\(\kappa\)B P65 enter nucleus, suggesting M1 polarization. HO-1 is a cytoprotective enzyme, which is the rate-limiting enzyme for heme decomposition. It decomposes heme into carbon monoxide, ferrous ions and biliverdin. HO-1 and its products regulate many biological processes, including inflammation, apoptosis, cell proliferation, fibrosis, and angiogenesis.25 We found that RA significantly increased the expression of HO-1 in macrophages, especially in LPS-stimulated M1 phenotype, and inhibited LPS-induced NF-\(\kappa\)B nuclear translocation. Furthermore, ZnPP, an inhibitor of HO-1, reversed the role of RA in inhibiting NF-\(\kappa\)B entry into the nucleus, suggesting that RA regulates macrophage polarization by HO-1. In addition, the NF-\(\kappa\)B inhibitor BAY11-7082 blocking NF-\(\kappa\)B signaling pathway has no impact on the role of RA regulating macrophages polarization. This result further shows that RA ultimately works by suppressing NF-\(\kappa\)B signal, but has no effect on other signal pathways. However, it is not clear whether there are other molecules signal between HO-1 and NF-\(\kappa\)B, which requires us to do more work in the future.

**Conclusion**

Our experiments showed that RA improves UC by inhibiting macrophages M1 polarization and favoring M2 polarization. In this process, the expression of HO-1 plays a crucial role by preventing NF-\(\kappa\)B nuclear translocation. Our data strongly suggest that reversing macrophage polarization can be used as a strategy for UC treatment and RA is an effective drug to cure UC by regulating macrophage polarization.
Availability of data and materials
The datasets used and/or analyzed during the current study are available from the authors Liping Zhang and Ping Mai on reasonable request.

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval
The present study was approved by Ethics Committee of Gansu Provincial People’s Hospital (no. syll20150001 Gansu, China).

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Informed consent
No human cases have been included in this study.

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