Clarithromycin Prevents the Progression of Dissected Aortic Aneurysm in the Experimental Study

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Research

Abstract

Background The cause of enlarged aortic dissection (AD) is associated with increased inflammatory cytokines such as IL-6. Clarithromycin (CAM) has been reported to inhibit inflammatory cytokines via suppressing NF-κB. we investigated whether or not CAM could prevent the enlargement of dissected aortic aneurysm.

Methods Male wild type mice (12 weeks of age) were infused with Angiotensin II and 3-aminopropinonitrile for two weeks for AD development. After two weeks, CAM (10 mg/kg/day) or saline was administered orally to the mice every day (CAM group: n=10, SAL group: n=10) with infusing Angiotensin II for more two weeks. After four weeks, the aortic diameter, macrophage infiltration, collagen and elastin quantities, and levels of inflammation related cytokines, were assessed.

Results The aortic diameter was significantly suppressed in the CAM group ($P < 0.01$). No rupture death was observed in the CAM group in contrast to 3 (30%) in the SAL group ($P = 0.07$). Clarithromycin significantly increased the infiltration of anti-inflammatory macrophages (20.8% vs. 2.8%, $P < 0.05$). Compared with the controls, the levels of IL-1β (332 pg/mL vs. 800 pg/mL, $P < 0.01$) and IL-6 (344 pg/mL vs. 727 pg/mL: $p < 0.05$) were significantly decreased, and the levels of IL-4 (2519 pg/mL vs. 1397 pg/mL, $P < 0.05$) and TGF-β (1649 pg/mL vs. 1134 pg/mL, $P < 0.05$) was significantly increased in the CAM group. The collagen area was increased (10.1% vs. 4.2%, $P < 0.05$) and expression of α-SMA (9.5% vs. 2.8%, $P < 0.05$) and α-actinin (16.3% vs. 4.0%, $P < 0.01$) were increased in the CAM group compared with the SAL group.

Conclusions CAM suppressed the progression of dissected aortic aneurysm through the anti-inflammatory and the existence of abundant collagen secreted including α-SMA, α-actinin positive cells.

Introduction

Aortic dissection (AD) which occurs when blood penetrates the intima and enters the media layer in the aorta is life-threatening disease [1,2]. Type A AD requires emergent surgery, on the other hand type B AD is generally managed by medical therapy such as anti-hypertensive treatment in the absence of complications [1,2]. However, the patients who suffered type B AD with enhanced inflammation sometimes present with aortic enlargement, thereby facing undesirable outcomes. General treatments for type B enlarged AD are basically open descending and thoracoabdominal aortic repairs by prosthetic graft replacement to prevent rupture of the aorta. However, descending and thoracoabdominal aortic repairs are high invasive procedures, consequently increasing the risk of postoperative severe complications such as paraplegia [1]. Therefore, less-invasive or non-surgical therapeutic approaches to treating type B AD are urgently required. Recently, the endovascular repair could be carried out to close entry of the dissection for type B AD before expansion of aortic diameter, however, the endovascular repair has some disadvantages including the adaptation, unclear prognosis and incomplete treatment because of residual re-entry and cost.

Aortic Dissection is caused by the destruction of extracellular matrix (ECM), such as elastin and collagen, which provide mechanical strength to the aortic wall. Increasing IL-6 and MMP-9, and decreasing TGF-β induced aortic expansion after AD [3-7].

Clarithromycin (CAM) is known to be an antibiotic among macrolides and has been reported to exert a range of biologic effects, including altering the expression of inflammatory factors and reducing MMP levels [7-9]. Furthermore, we reported that CAM found to inhibit NF-κB phosphorylation, which is known to be a pivotal signal of the inflammation pathway, and resulting showed suppression of aortic aneurysm formation and rupture [7,10]. It has been reported clarithromycin has anti-inflammatory effects such as suppression of NF-κB phosphorylation, IL-6, MMP-2 and -9 [7]. We therefore hypothesized that the administration of CAM might inhibit the dilatation of dissected aortic aneurysm.

In the present study, we investigated whether or not orally administered CAM was able to prevent the enlargement of dissected aortic aneurysm using AD induced mice.

Materials And Methods

AD Model and CAM Prescription

Wild type mouse model of angiotensin II (Ang II) and 3-aminopropionitrile (BAPN)-induced AD was used in this study. Male mice (12 weeks old) were infused with 1000 ng/kg/min Ang II (Calbiochem, San Diego, CA, USA) for 28 days and 300mg/kg/day BAPN (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) for 14 days. Ang II was infused using Alzet osmotic pumps (Model 2004; DURECT, Cupertino, CA, USA) and BAPN was infused using Alzet osmotic pumps (Model 2002; DURECT, Cupertino, CA, USA) as described previously [11]. Mice were anesthetized with isoflurane delivered using a calibrated vaporizer equipped with an induction chamber and a nose cone. After induction, the concentration was reduced to 2% to maintain anesthesia. Pumps were implanted subcutaneously into the back in the prone position through a small incision that was closed with sutures after implantation.

After two weeks from pump implantation, the male wild type mice were divided randomly into two groups: a control group, containing mice with Ang II infusion and oral saline administration (SAL group, n=10); and a CAM-treated group, containing mice with Ang II infusion and oral CAM administration (CAM group, n=10). Mice were administered CAM (10 mg/kg/day) or saline via a gastric tube every day. CAM was dissolved with saline at 1.2×10^{-4} µg/µL and administered at a volume of 200 µL every day, and the SAL group received the same amount of saline. After the implantation of pumps, the administration of CAM or saline was simultaneously started (Figure 1A).

Echography

Before the implantation of pumps and every week after the implantation, echocardiography (10MHz) (GE Healthcare, Chicago, IL, USA) was performed, and the aortic diameters from the thoracoabdominal to terminal aorta were measured. Mice were anesthetized with isoflurane, and echography was performed

on the abdomen in the supine position to confirm the existence of AD and measure the maximum diameter of the aorta and the existence of aortic aneurysm (AA) (Figure 1B). A commonly used clinical standard to diagnose AA is an increase in aortic diameter of more than 50%, so AAs were defined as dilation to at least 1.5 times the pre-implantation diameter.

Measurement of Aortic Diameter and Specimen Preparation

See Supplementary Data.

Elastica van Gieson Staining and Masson's Trichrome staining

See Supplementary Data.

Immunofluorescence Staining

See Supplementary Data.

Expression of Protein in the Aortic Wall

See Supplementary Data.

Statistical Analyses

Data analyses were performed with the IBM SPSS software program, version 25 (IBM, Armonk, NY, USA). The results were expressed as the mean ± standard error of the mean. The incidence of rupture of AD was assessed using the Kaplan-Meier method, and statistical comparisons were performed using the log-rank test. Aortic diameters of mean values were performed by a two-way factorial analysis of variance (ANOVA). Groups were compared using unpaired t-tests. Statistical significance was defined as $P < 0.05$.

Results

Aortic Diameter

In echo studies, the maximal aortic diameters were 1.09 ± 0.02 mm in the CAM group and 1.14 ± 0.02 mm in the SAL Group ($P = 0.15$) before prescription, 1.50 ± 0.06 mm and 1.58 ± 0.08 mm ($P = 0.39$) after 1 week, 1.85 ± 0.10 mm and 1.96 ± 0.10 mm ($P = 0.46$) after 2 weeks, 1.93 ± 0.08 mm and 2.37 ± 0.20 mm ($P = 0.07$) after 3 weeks, and 1.79 ± 0.10 mm and 2.67 ± 0.24 mm after 4 weeks ($P < 0.01$), respectively. Aortic diameter obviously increased between 2 weeks and 4 weeks in SAL Group ($P < 0.05$), on the other hand, CAM suppressed aortic expansion between 2 weeks and 4 weeks ($P = 0.28$) (Figure 2A). The incidence of the development of the descending aortic aneurysm in both groups was 100%. A Larger aortic aneurysm was observed in the descending aorta in the SAL group. After two weeks, three deaths due to rupture of dissected aortic aneurysm occurred in the SAL group, while no such deaths were observed in the CAM group. Figure 2B shows representative images of the aortas from both groups. Figure 2C represents the free-from aortic rupture rate ($P = 0.07$).

Evaluation of Aortic Collagen and TGF-β

The EVG-stained, Masson's Trichrome-stained and immunofluorescence sections from the thoracoabdominal aortas of the CAM group showed that abundant collagen tissues in defect of medial layer was relatively well-maintained in the aortic wall, but sections from the SAL group showed the irregular and thin collagen tissues in defect of media and aneurysm formation (Collagen: 10.1 ± 2.1% vs. 4.2 ± 0.9 %, $P < 0.05$) (Figure 3ABC). Immunofluorescence staining revealed TGF-β1 were detected abundantly associated with Arginase-1 positive macrophage in the CAM group (TGF-β1: 0.80 ± 0.12 % vs. 0.13 ± 0.03 %, $P < 0.05$) (Figure 4AB).

Immunostaining for Macrophages

Immunofluorescence staining revealed the prolific presence of iNOS-positive macrophages in the adventitia and media of the aortic walls in the SAL group, while Arginase-1-positive macrophages were detected abundantly in the CAM group. The infiltration of proinflammatory macrophages was inhibited by CAM administration (Figure 5A). Figure 5B showed counts of the iNOS and Arginase-1 staining area as a ratio of the DAPI (iNOS: 7.2 ± 2.7% vs. 27.8 ± 6.5 %, $P = 0.02$, Arginase-1: 20.8 ± 7.0 % vs. 2.8 ± 1.1 %, $P < 0.05$).

Immunostaining for Non-muscle Alfa-actinin and Alfa-smooth muscle actin

Immunofluorescence staining also revealed the development of non-muscleα-actinin and α- smooth muscle actin (SMA) in the media of the aortic walls, especially defect of smooth muscle cell, in the CAM group. (Figure 6A). Figure 6B showed counts of the α-actinin and α-SMA staining area as a ratio of the DAPI (α-actinin: 3.6 ±0.6 % vs. 22.5 ± 6.0 %, $P < 0.01$, α-SMA: 12.0 ± 1.3 % vs. 3.4 ± 0.6 %, $P < 0.05$).

Expression of AD-Related Proteins

An examination of the protein levels in aortic tissues revealed that CAM significantly reduced the expression of IL-1β (CAM vs. SAL; 331.6 ± 46.9 pg/mL vs. 799.6 ± 124.1 pg/mL : $P < 0.01$) and IL-6 (334.4 ± 72.7 pg/mL vs. 726.5 ± 52.5 pg/mL : $P < 0.01$) (Figure 6A). CAM significantly increased the expression of IL-4 (2518.5 ± 1128.8 pg/mL vs 1396.8 ± 239.0 pg/mL : $P < 0.05$) and TGF-β (1649.1 ± 161.7 pg/mL vs 1133.8 ± 139.1 pg/mL : $P < 0.05$) but not statistically the expression of IL-10 (29.9 ± 16.1 pg/mL vs 4.5 ± 0.5 pg/mL: $P = 0.19$)) in ELISA analysis. (Figure 7)

Discussion

In the present study, we showed that orally administered CAM suppressed the progression of dissected aortic aneurysm, suppressed inflammatory reactions and induced anti-inflammatory reactions and collagen synthesis in the aortic wall. There has been anti-hypertensive drug and β blockade for prevention aortic enlarged AD. It has been reported losartan suppressed aortic enlargement and dissection in patients with Marfan Syndrome. Losartan, an angiotensin-II receptor blocker (ARB) that has previously

demonstrated TGF-β antagonism, has been studied [12] for AD to prevent aortic enlargement, therefore, there are no definitive drugs for AD.

The previous studies showed AD were induced by TGF-β antibodies injection with administration of Angiotensin II or a model of the combination with BAPN administration in mice [13-15]. In those models, the BAPN with Angiotensin II mice tended to develop aortic dissection, then we could induce the AD model by using BAPN and Angiotensin II to the wild mice for two weeks [16,17]. All mice could confirm the development of aorta dissection with aortic echography by two weeks, and then were gave Angiotensin II for the next two weeks for exposing them under high blood pressure for induction of dissected aortic aneurysm. To investigate whatever CAM might have suppressed the progression of induced dissected aortic aneurysm, we administered CAM orally.

It has been reported that CAM is not only a macrolide antibiotic but has anti-inflammatory property. Although, this mechanism has not been clear, CAM can suppress NF-kappa B activation, induction of MMPs and inflammatory cytokines. We have already reported that CAM prevents atherosclerotic aortic aneurysm formation in mice by suppression of NF-kappa B activation, inflammatory cytokines, inflammatory macrophages accumulation and MMP- 2 and -9 activation [7].

Some studies reported that cause of enlargement of dissected aortic aneurysm could be inflammatory cytokines activation including IL-6 [3,18], and decreasing TGF-β [6,19]. CAM redressed abnormalities of these cytokines and chemokines [20], so we thought that CAM might suppressed the enlargement of the dissected aortic aneurysm. In this study, increase of collagen and TGF-β were confirmed in dissected walls in CAM group, and the structures including alignment of collagen in the dissected vascular wall were more regular structures than in SAL group. Furthermore, α-SMA and α-actinin were confirmed around their collagen. The strength of the aortic wall is defined by the quantities of extracellular matrices, then collagen expression in the dissected wall might contribute to make the aortic wall strong [21-23].

Alpha-SMA expression is a hallmark of the mature myofibroblast and has proven to be a reliable marker for identifying vascular smooth muscle cells during vascular development and vascular diseases, and myofibroblasts during wound healing [25]. Non-muscle α-actinin is a cytoskeletal actin-binding protein and has a number of important functions such as maintenance of cell's internal scaffold, provision of mechanical stability, locomotion, intracellular transport of organelles, as well as chromosome separation in mitosis and meiosis [26]. The phenotype of myofibroblast in expressing α-SMA and producing ECM compound is regulated by TGF-β and myofibroblasts which changed to smooth muscle-like cells express α-actinin [27-29]. Figure 6 showed that α-SMA and α-actinin positive cells were developed in the adventitia. The adventitia includes mainly fibroblast, then TGF-β binding to TGF-β receptor on the fibroblast leads the synthesis of ECM such as collagen. Schriefl and colleagues has reported that α-SMA appeared to be responsible for the newly produced collagen, which protected region of the dissected wall [30]. In this study, CAM induced TGF-β expression, which might induce fibroblast to myofibroblast. The expression of α-SMA and α-actinin was confirmed with a regular structure in the collagen. The development of the stromal cells including myofibroblasts with collagen in the media suggests increase

the strength of the aortic wall. Thus, these phenomena might contribute suppress the enlargement of dissected aortic aneurysm.

Some limitations associated with the present study warrant mention. First, the CAM dose was lower than the previous studies [7]. We selected a dosage of 10 mg/kg/day in the present study. Though this dose was as high as that generally administered for antibiotic treatment clinically, we did not evaluate CAM dose in the aortic tissue and the blood, then further studies will be required to determine the most effective dosage with the fewest, mildest side effects. Second, the BAPN dose was also higher than in most previous reports [13,17]. Regarding BAPN dosage, although 150 mg/kg/day has been used in some reported studies, but used a dosage of 300 mg/kg/day because we wanted to promote the development of AD at the high rate. This dosage led to marked AD formation and a spectacularly higher rate of dissection than 150m mg/kg/day, so future studies may want to consider using a lower dosage of BAPN. Third, CAM was administered within two weeks after development of aortic dissection, then, it was unknown when CAM was started after aortic dissection development. Finally, because the part of the aorta used for protein analyses also contained part except aortic aneurysm, it may reflect not only aortic aneurysm.

In conclusion, our findings here suggest that CAM can prevent the progression of dissected aortic aneurysm via M2 macrophage accumulation and anti-inflammatory cytokines such as IL-4 and TGF-β. Further investigations will be required in order to adapt clinical therapy of CAM for the prevention of dissected aortic aneurysm expansion.

Declarations

References

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Figures
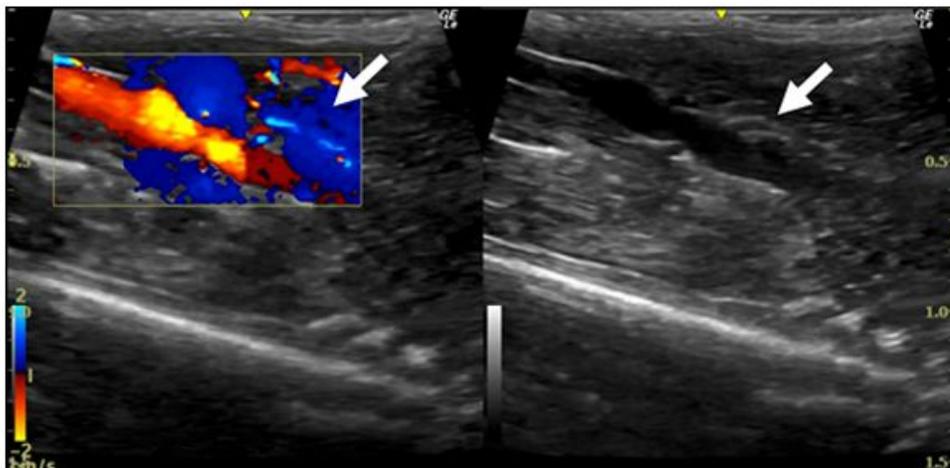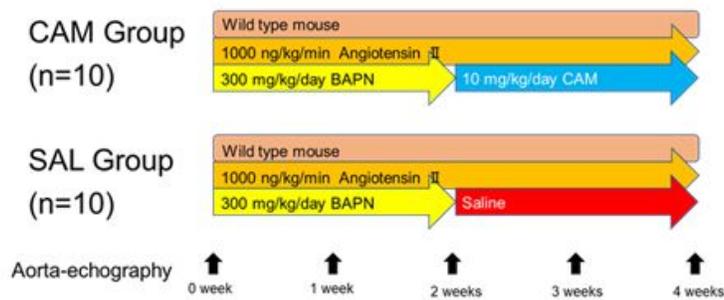
Figure 1

1A. Study protocol. 1B. Echography showed aortic dissection. The arrow indicates a false lumen.

Figure 2
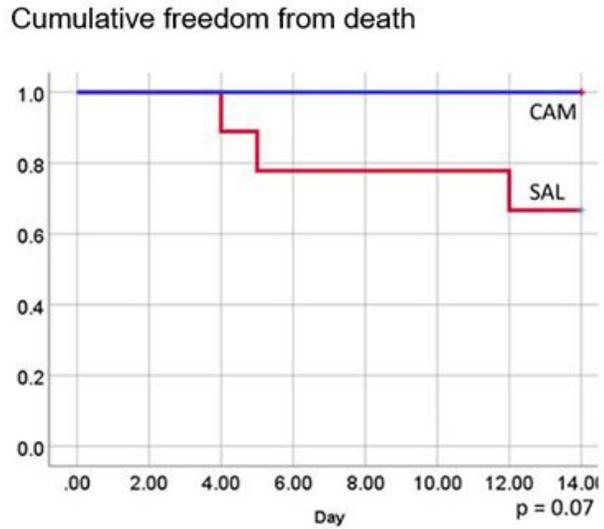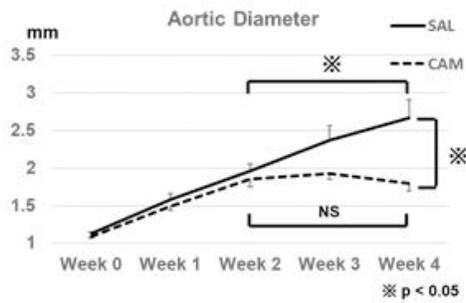
2A. Aortic diameter: Echography of the aorta showed that the expansion of the aortic diameter was significantly attenuated in the CAM group. 2B. Macroscopic findings Macroscopic observation showed a larger and purplish aortic aneurysm in the SAL group, while a white and secure aortic aneurysm was observed in the CAM group. 2C. Cumulative rate of freedom from death No deaths due to aneurysm rupture were observed in the CAM group. In contrast, 3 deaths due to aneurysm rupture were observed in the SAL group after 14 days.

Elastica van Gieson staining

Immunofluorescence staining

Masson's Trichrome staining

Immunofluorescence staining with DAPI

SAL   CAM   SAL   CAM

Collagen Area

■ SAL
□ CAM

※ p < 0.05
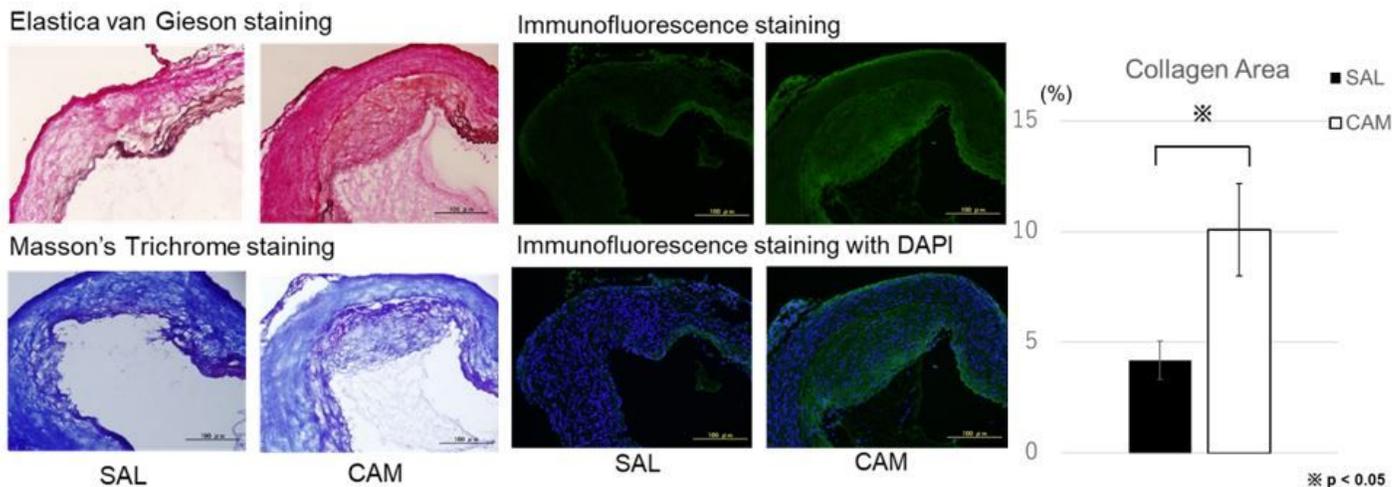
Figure 3

3ABC. Evaluation of collagen Elastica van Gieson staining and Immunofluorescence staining showed that collagen tissues were more abundant in the CAM group than in the SAL group.
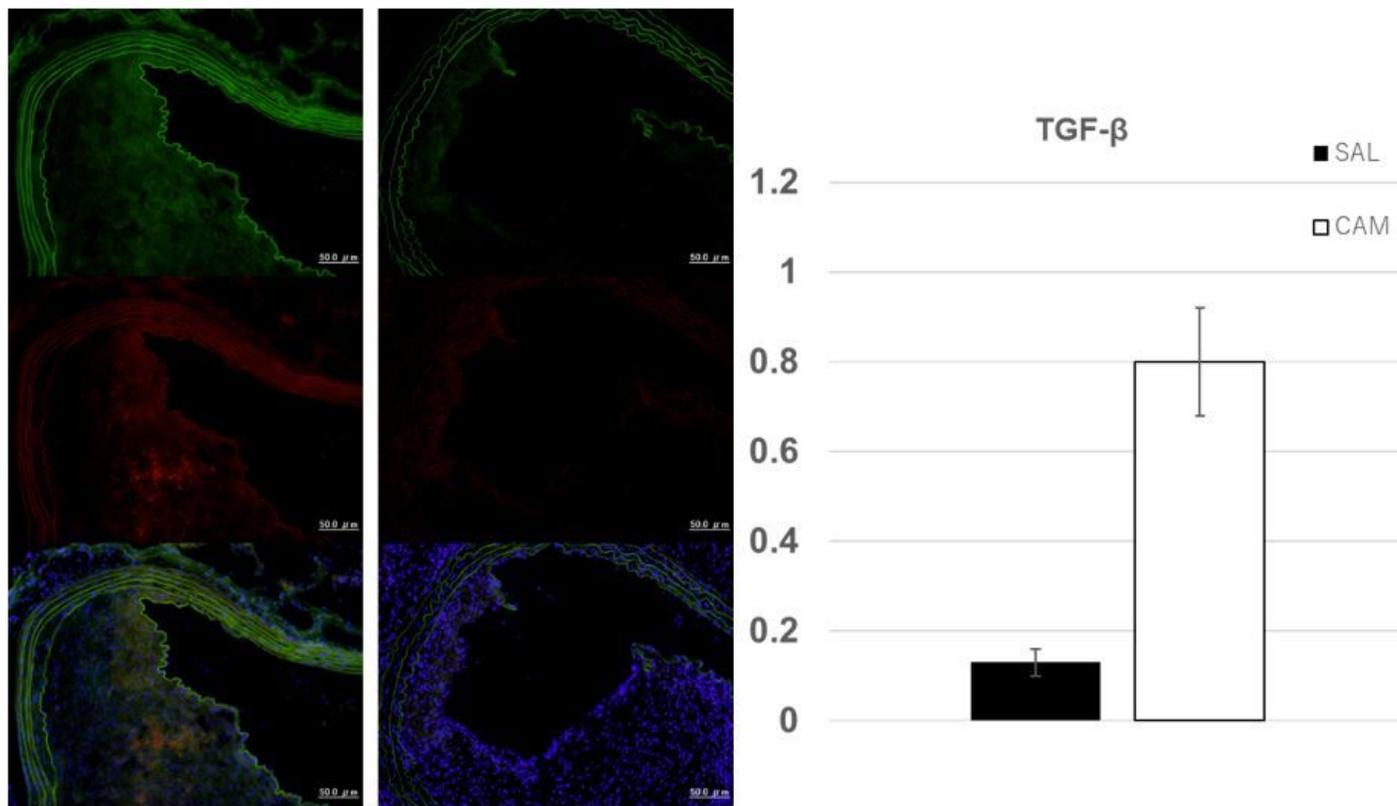

TGF-β

■ SAL
□ CAM

Figure 4

4AB. Evaluation of TGF-β Clarithromycin increased the accumulation of TGF-β, which was associated with the accumulation of M2 macrophages.
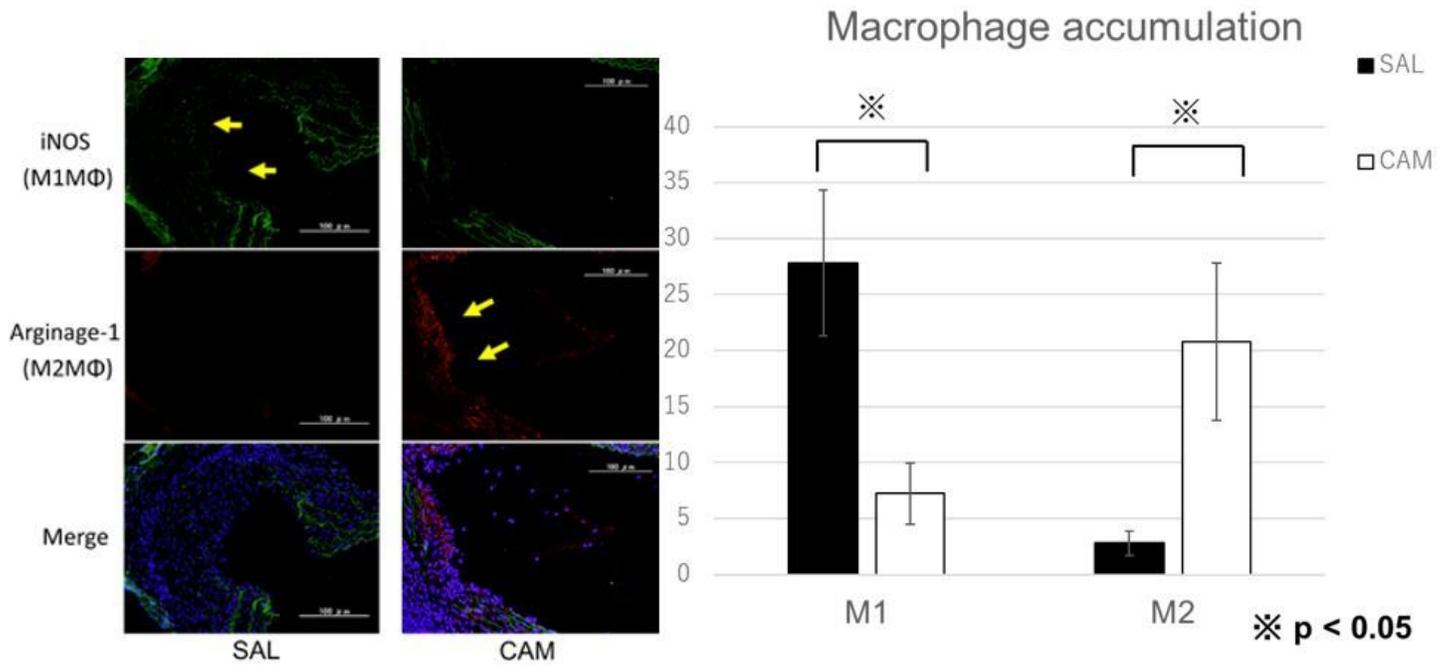
Figure 5

5AB. Evaluation of macrophages CAM attenuated the accumulation of iNOS-positive M1 macrophages and increased the accumulation of Arginase-1-positive M2 macrophages.
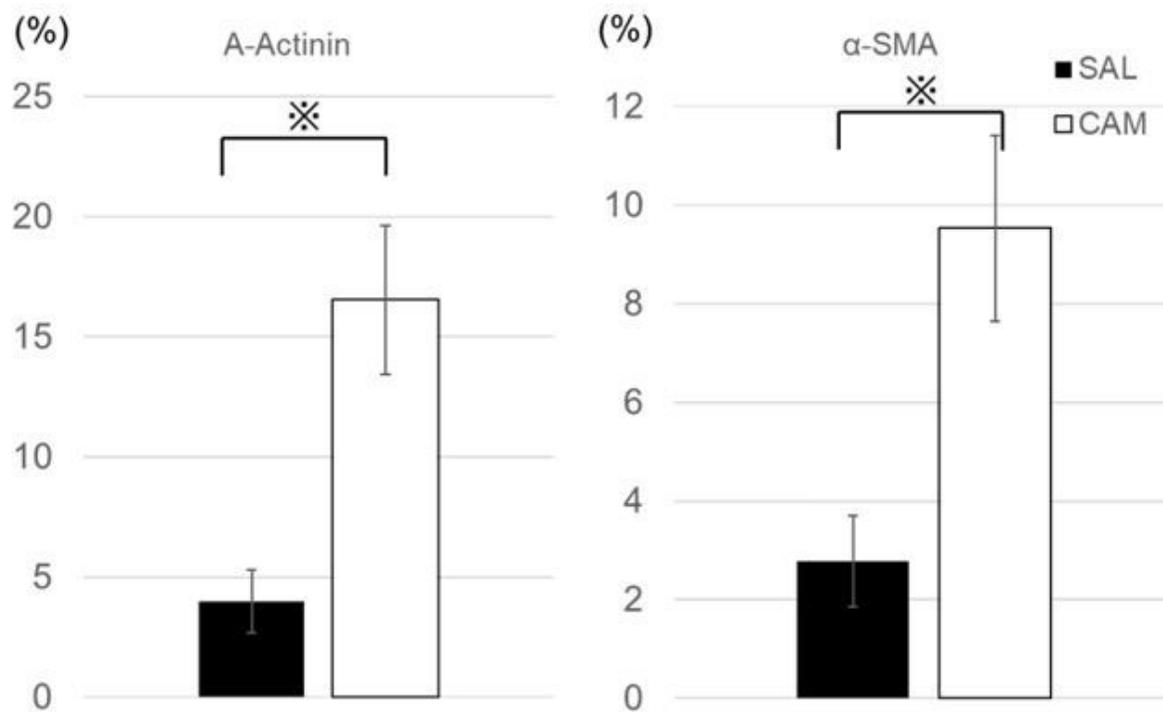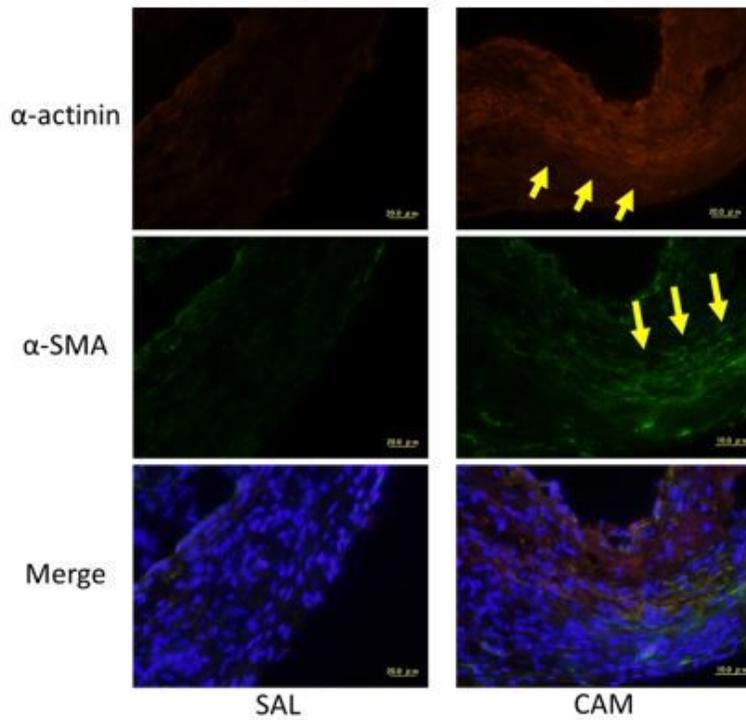
Figure 6

6AB. Evaluation of α-SMA and α-actinin CAM obviously increased the accumulation of α-actinin and α-SMA.
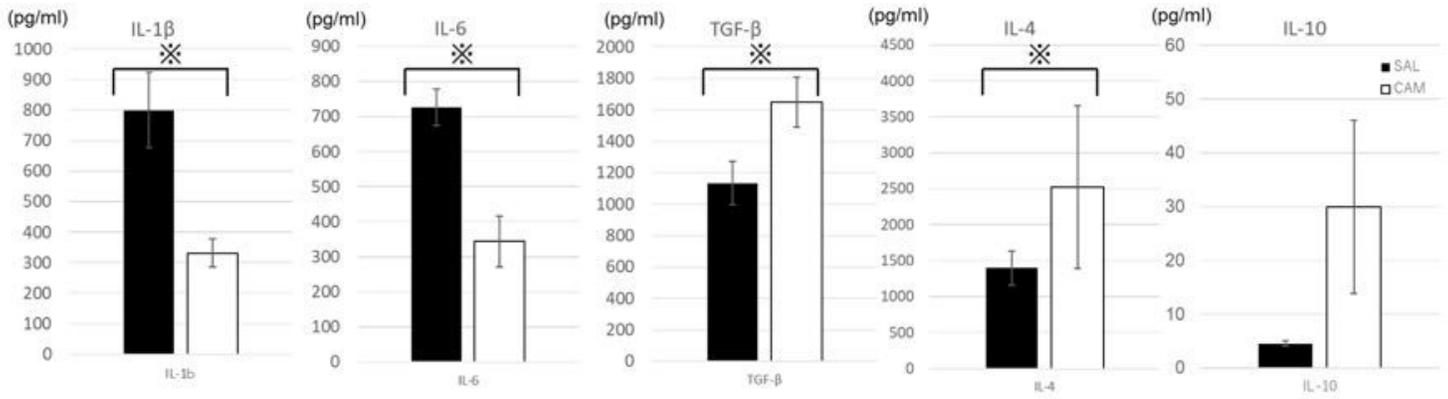
Figure 7

Protein analysis by ELISA The ELISA analysis demonstrated that clarithromycin significantly suppressed the expression of IL-1β and IL-6, and increased the expression of IL-4 and TGF-β.