Immunopathologia Persa
http immunopathol.com
DOI:10.34172/ipp.2025.42741

The immunomodulatory effect of ivermectin on rat vasculitis model

Teeba Sarem Sadeq[1*], **Nadia H Mohammed**[2], **Inam Sameh Arif**[1]

[1]Department of Pharmacology and Toxicology, College of Pharmacy, University of Mustansiriyah, Baghdad, Iraq
[2]Department of Microbiology and Immunology, College of Medicine, University of Mustansiriyah, Baghdad, Iraq

***Correspondence to**
Teeba Sarem Sadeq,
Email:
teeba_sarim@uomustansiriyah.edu.iq

Received 28 Aug. 2024
Accepted 28 Nov. 2024
ePublished 20 Jan. 2025

Keywords: Vasculitis, Adaptive immune system, Ivermectin, Interleukin-6, C-reactive protein, Myeloperoxidase ANCA, Immunohistochemistry

Abstract

Introduction: Vasculitis is the inflammation of the blood vessel walls and tissues, affecting single organs or multiple systems and is often a hidden factor in numerous disorders. Different pathogens induce innate and adaptive immune system cells to release pro-inflammatory cytokines. These processes lead to the production of proteolytic enzymes, oxidative stress, vascular homeostasis disruption, and destruction of endothelial cells.
Objectives: This study aims to investigate the immunomodulatory effects of ivermectin on the rat vasculitis model.
Materials and Methods: This experimental animal study included 33 male Wistar albino rats which were divided into five groups. The disease was induced in all groups except group I (control); rats of this group received the dissolving vehicles only. Group II (vasculitis induction group) received ovalbumin and lipopolysaccharide (LPS). Group III, group IV and group V (ivermectin groups) were pretreated with 0.5 mg/kg, 1 mg/kg and 1.5 mg/kg of ivermectin, respectively, for seven days, followed by vasculitis induction using ovalbumin and LPS.
Results: This study showed a significant reduction ($P<0.001$) in serum interleukin-6 (IL-6), C-reactive protein (CRP) and myeloperoxidase (MPO) anti-neutrophil cytoplasmic antibody (ANCA) levels in rats who received ivermectin compared to the disease group. Immunohistochemistry staining of Toll-like receptor 4 (TLR4) showed a decrease in the expression of this receptor in ivermectin groups.
Conclusion: Ivermectin modulates inflammatory reactions by inhibiting inflammatory marker expression. Therefore, ivermectin can be effective in protecting against the disease.

Citation: Sadeq TS, Mohammed NH, Arif IS. The immunomodulatory effect of ivermectin on rat vasculitis model. Immunopathol Persa. 2025;x(x):e42741. DOI:10.34172/ipp.2025.42741.

Introduction

Vasculitis is inflammation of the blood vessel walls and vascular tissues. The disease may manifest as either a single organ or a multi-systemic condition; in some cases, symptoms are absent until organ damage reaches the end stage (1). Vasculitis is classified depending on the etiology, pathogenesis, location and blood vessel size (2). Certain factors enhance disease susceptibility, such as age, infection, environment, hormones, genetics, and drugs (3). Pathogenic immune mediators trigger vasculitis immunopathogenesis, including anti-neutrophil cytoplasmic antibody (ANCA)-mediated vasculitis, immune complexes, and T-cell-mediated immune responses (4). ANCA is related to small vessel vasculitis and is categorized by two specific antigen targets; myeloperoxidase (MPO) and proteinase 3 (PR3) (5). Numerous pathogens stimulate innate immune cells and secretion of pro-inflammatory cytokines, followed by the activation and priming of neutrophils and formation of neutrophil extracellular traps (NETs) (6). The activation of pattern recognition receptors, particularly Toll-like receptors (TLRs), in response to a bacterial toxin such as lipopolysaccharide (LPS) stimulates the innate immune response and enhances the secretion of interleukin-6 (IL-6) from numerous cells (7). The latter is essential in activating the adaptive immune system. IL-6 activates complement pathways and acute phase proteins, like C-reactive protein (CRP) (8,9). CRP has the ability to bind to foreign molecules and opsonize them by macrophages (10,11). Sequences leading to oxidative stress, tissue necrosis, and cell destruction can cause vascular homeostasis loss, vessel obstruction, ischemia, and hemorrhage (12). The Food and Drug Administration (FDA) approved ivermectin as an anti-parasitic drug (13).

Key point

The recent study explores the impact of ivermectin in modulation of inflammation by reducing the stimulation of the immune system in response to various pathogens.

Ivermectin shows multiple antiviral activities against human immunodeficiency virus (HIV) (14) and SARS-CoV-2 (15).

Objectives

The present study aimed to investigate the immunomodulatory effects of ivermectin in a vasculitis disease model in rats.

Materials and Methods

Drug and chemicals

Ivermectin crude powder (CAS number 70288-86-7, purity 98%) was acquired from Meryer, Shanghai, China. Ovalbumin powder (CAS number 9006-59-1) was acquired from Macklin, Shanghai, China. LPS powder, Escherichia coli O55:B5 (CAS number 93572-42-0) was acquired from Solarbio, Beijing, China.

The enzyme-linked immunosorbent assay kits

The enzyme-linked immunosorbent assay (ELISA) serum kits utilized in the current study were IL-6 (catalog number; SEA079Ra), and CRP (catalog number: SEA821Ra) that were acquired from Cloud-clone crop, USA. MPO ANCA (catalog number: CSB-E08675r) was acquired from CUSABIO, USA.

Ivermectin preparation

Ivermectin is a hydrophobic drug. A mixture of propylene glycol and glycerol (60:40% v/v) was used to dissolve the powder (16). After the ivermectin dose was adjusted for rats according to the human dose equation, a stock solution containing 10 mg of ivermectin was prepared using a magnetic stirrer device for 10 minutes to improve the dissolution of the powder. Ivermectin groups were administered daily intraperitoneal injections of the drug for one week, and doses were adjusted depending on the rats' average weights.

Vasculitis induction

Experimental rats received nine doses of ovalbumin (7.5 mg/kg) from day 8 to day 32 every third day, followed by three doses of LPS (2 mg/kg) on the 27th, 30th, and 33rd days (17). Animal groups received intraperitoneal doses after adjusting them according to the rats' average weights.

Experimental design and animals grouping

This animal study included 33 male Wistar albino rats aged approximately six weeks and a weight range from 120 g to 130 g. Rats were acquired from the animal house, Iraqi center for cancer and medical genetics research, Mustansiriyah University. The experimental animals were acclimatized using a sterilized cage with a well-ventilated environment, unrestricted to food and water and exposed to 12/12 hours of light/dark cycle, in the animal house at the college of pharmacy, Mustansiriyah University.

All the experiment groups received ovalbumin and LPS to induce the disease except the control group. In group I (n=6), control group rats received a mixture of propylene glycol and glycerol (60:40% v/v) daily for one week, followed by normal saline injection by intraperitoneal route as the exact timeline of the disease induction group. In Group II (n=6), induction group, rats received a mixture of propylene glycol and glycerol (60:40% v/v) daily for one week, followed by nine doses of ovalbumin (7.5 mg/kg) from day 8 to day 32 every third day. Then, three doses of LPS (2 mg/kg) on the 27th, 30th, and 33rd days, doses were injected by intraperitoneal route. In groups III, IV and V (n=7 in each group, the ivermectin groups), the rats received ivermectin dissolved in propylene glycol and glycerol mixture daily for one week. Group III, rats were injected with 0.5 mg/kg, group IV with 1 mg/kg, and finally, group V with 1.5 mg/kg, followed by nine doses of ovalbumin (7.5 mg/kg) from day 8 to day 32 every third day. Then, three doses of LPS (2 mg/kg) were administered on the 27th, 30th, and 33rd days; all doses were injected using the intraperitoneal route.

Serum markers measurement

Blood samples were obtained by myocardial puncture using a 5 mL syringe at the end of the experiment. The samples were placed in a separate gel tube for 15 minutes to allow clotting and centrifuged for 15 minutes at a speed of 3000×. Clear serum was obtained to measure serum markers (IL-6, CRP, and MPO ANCA) using the ELISA technique. The microplate has been pre-coated with an antibody specific to rat serum antigens. Kit components and samples were placed at room temperature; the standard solution was prepared by adding 1 mL of standard diluent buffer with gentle shaking. The stock solution was diluted to prepare a series of tubes containing 0.5 mL of the standard diluent with different concentrations; the last tube with standard diluent was blank. Then, we added 100 µL of diluent to the empty wells using a micropipette. The assay procedure was conducted by adding appropriate dilutions of 100 µL of standard, blank, and samples to each of the wells, covered and incubated for an hour at room temperature. Then, snap the plate onto absorbent paper to remove the unbound sample component. Biotinylated detection antibody working solution was added to each well, incubated for an hour and then washed. A concentrated horseradish peroxidase (HRP) conjugate working solution of 100 µL was added to each well, incubated for 30 minutes, and then rinsed. In a dark place, 90 µL of substrate tetramethylbenzidine solution was added to each well and left for 15 minutes, which was followed by adding 50 µL of stop solution (acid) and tapping it to allow mix. The liquid turned yellow for positive sample wells. A microplate reader at 450 nm wavelength was used to measure the optical density value of inflammatory markers.

Immunohistochemistry analysis and scoring

Rats received intraperitoneal injection of ketamine (100

mg/kg) and xylazine (10 mg/kg) (18). Lung tissue was isolated and fixed in a 10% neutral buffered formalin saline for 24 hours and then dehydrated in ethanol serial concentrations. This procedure was followed by 2 hours of xylol and a paraffin bath at 57 ˚C melting point to prepare 4 µm sliced sections. Sections were stained with hematoxylin and eosin. Sections for immunohistochemistry (IHC) were deparaffinized in xylene and rehydrated in a series of alcohols. The next steps were heat-induced epitope retrieval, peroxidase activity blocking and incubation with a protein blocking solution. Primary antibody overnight at 4 ˚C. Sections were incubated with an anti-human rabbit monoclonal antibody against the Toll-like receptor 4 (TLR4) antibody (Abcam, United States). Then, a secondary antibody was applied for 30 minutes before the sections were covered with a chromogen substrate (1% diaminobenzidine). Hematoxylin counterstain for 3 minutes gives a blue color around the tissues to identify the cell changes, improve visualization and distinguish the cell from the other cells. Finally, dehydration and covering the tissue sections with a glass coverslip for evaluation under the light microscope (19).

A pathologist evaluated lung tissue sections to identify TLR4 in the cell membrane. The percentage of positively stained cells was determined and scored as: ≤5% [0]; 6-25% [1]; 26-50% [2]; 51-75%[3]; 76-100% [4]. The staining intensity was scored into four categories depending on the color of the immune reaction. Negative→0, light brown→1, brown→2, dark brown→3. The final score of TLR4 was obtained by multiplying the percentage of positive stained cells and the staining intensity. A final score of 0-2 was considered a negative expression (-, noted as 0); 3–4: weak positive expression (+, noted as 1); 6-8: moderate positive expression (++, noted as 2); 9-12: strong positive expression (+++, noted as 3) (20).

Statistical analysis

Statistical Packages for Social Sciences (SPSS) version 23 was used for data analysis. Means and standard deviations (SD) were used to express the quantitative variables. Statistical significance was determined by one-way analysis of variance (ANOVA) and a post hoc test. A *P* value less than 0.05 represented statistical significance.

Results

Effect of ivermectin on serum IL-6

IL-6 biomarker levels are displayed in Table 1 across the study groups. A significant elevation ($P<0.001$) in IL-6 level in group II (disease group) compared to group I (control group). Results of III, IV and V groups (ivermectin groups) show a significant decrease in IL-6 levels compared to group II ($P<0.001$).

Effect of ivermectin on serum CRP

CRP levels across the study groups are illustrated in Table 2. The result revealed a significant elevation ($P<0.001$) in CRP levels in group II (induction group) compared to group I (control group). The results of III, IV, and V groups (ivermectin groups) show a significant decrease ($P<0.001$) in CRP levels compared to group II.

Effect of ivermectin on serum MPO ANCA

MPO ANCA levels are presented in Table 3 across the study groups. The results showed a significant elevation ($P<0.001$) in MPO ANCA levels in group II (induction group) compared to group I (control group). Results of III, IV, and V groups (ivermectin groups) show a significant decrease ($P<0.001$) in MPO ANCA levels compared to group II.

Table 1. The effect of ivermectin on interleukin-6 levels in male rats' sera among the study groups

Group	Rats (n)	IL-6 (pg/mL) Mean ± SD	*P* value[a]
I	6	201.3 ± 28.9	
II	6	556.4 ± 89.4	
III	7	362.7 ± 10.6	<0.001
IV	7	208.0 ± 9.2	
V	7	99.8 ± 5.4	

IL-6: Interleukin-6. Group I: control, group II: induction, group III: Ivermectin 0.5 mg/kg, group IV: Ivermectin 1 mg/kg, group V: Ivermectin 1.5 mg/kg.
[a]One-way ANOVA test was conducted to determine the statistical significance of differences among the groups, with a *P* value of less than 0.001 indicating significant variations.

Table 2. The effect of ivermectin on serum C-reactive protein levels in male rats among the study groups

Group	Rats (n)	CRP (mg/L) Mean ± SD	*P* value[a]
I	6	5.5 ± 0.5	
II	6	23.3 ± 3.3	
III	7	16.1 ± 1.0	<0.001
IV	7	5.2 ± 0.3	
V	7	4.9 ± 0.3	

CRP: C-reactive protein. Group I: control, group II: induction, group III: Ivermectin 0.5 mg/kg, group IV: Ivermectin 1 mg/kg, group V: Ivermectin 1.5 mg/kg.
[a]One-way ANOVA test was conducted to determine the statistical significance of differences among the groups, with a *P* value of less than 0.001 indicating significant variations.

Table 3. The effect of Ivermectin on serum myeloperoxidase ANCA levels in male rats among the study groups

Group	Rats (n)	MPO ANCA(U/mL) Mean ± SD	*P* value[a]
I	6	7.8 ± 1.0	
II	6	26.2 ± 2.3	
III	7	10.0 ± 2.0	<0.001
IV	7	7.6 ± 1.4	
V	7	7.6 ± 2.3	

MPO ANCA: myeloperoxidase anti-neutrophil cytoplasmic antibody. Group I: control, group II: induction, group III: Ivermectin 0.5 mg/kg, group IV: Ivermectin 1 mg/kg, group V: Ivermectin 1.5 mg/kg.
[a]One-way ANOVA test was conducted to determine the statistical significance of differences among the groups, with a *P* value of less than 0.001 indicating significant variations.

IHC of TLR4 expression

The results of TLR4 expression in inflammatory cells are displayed in Table 4 and Figure 1, depending on the proportion of cell staining multiplied by the staining intensity. However, non-specific staining of other cells, such as congested red blood cells, lymphocytes and fibrin, was observed. IHC staining results of lung tissue sections of the experiment groups are illustrated in Figure 2.

Discussion

Vasculitis is inflammation of the vascular walls and is regarded as a hidden factor for various illnesses (21). In ivermectin groups, the serum IL-6 level was significantly reduced compared to the disease group. A similar outcome was reported in a previous study by Zhang et al, where animals were pre-treated with a single dose of ivermectin prior to LPS injection (22). IHC evaluation of lung tissue displayed a reduction in the expression of TLR4 in ivermectin groups compared to the disease group. Ivermectin modulates the innate immune response to various pathological molecules by decreasing the expression of pattern recognition receptors mediated through the TLR4 pathway, co-receptor CD14, nuclear factor kappa-B (NF-κB) and interferon-β signaling pathways' activation. These pathways initiate further stimulation of antigen-presenting cells, including macrophage and dendritic cells and the release of pro-inflammatory cytokines such as IL-6 and tumor necrosis factor-α (23,24).

Ivermectin impedes disease severity and protects against the damage of alveolar tissue by reducing the inflammation reaction. The elevated levels of inflammatory cytokines are related to an increase in the

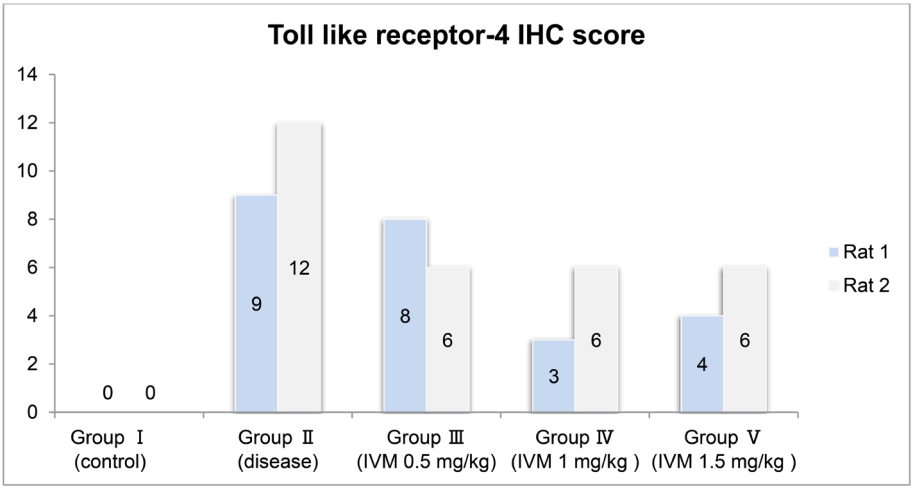

Figure 1. Toll like receptor-4 expression among the study groups.

Table 4. Immunohistochemistry scores for Toll-like receptor 4 expression in male rats' lung tissue sections among experimental study groups

Group	Rat	Staining intensity	Positive stained cells	Final score
Group I (control group)	1	3	0	0 -, score 0
	2	2	0	0 -, score 0
Group II (disease induction group)	1	3	3	9 +++, score 3
	2	3	4	12 +++, score3
Group III (ivermectin group) 0.5 mg/kg	1	2	4	8 ++, score 2
	2	3	2	6 ++, score 2
Group IV (ivermectin group) 1 mg/kg	1	3	1	3 +, score 1
	2	3	2	6 ++, score 2
Group V (ivermectin group) 1.5 mg/kg	1	2	2	4 +, score 1
	2	3	2	6 ++, score 2

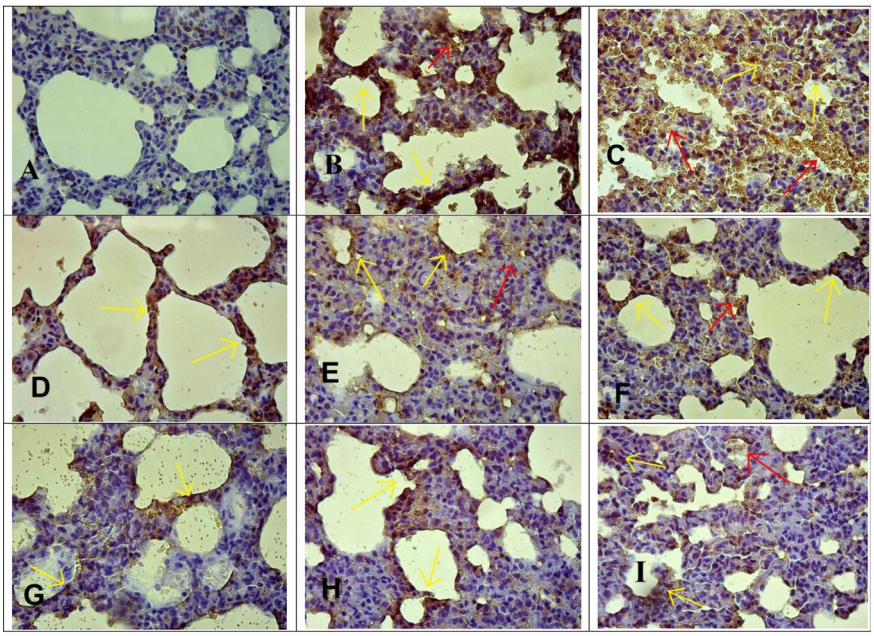

Figure 2. Immunohistochemistry evaluation of experimental groups rat lung tissue. (A) group I (control group) showing negative expression of TLR4 (-, score 0). A, B, D, F, H magnification 100×. C, E, G, I magnification 400×. (B)(C) group II (disease induction group) showing positive strong expression of TLR4 (+++, score 3). (D)(E) group III (Ivermectin 0.5 mg/kg group) showing moderate positive expression of TLR4 (++, score 2). (F)(G) Group IV (Ivermectin 1 mg/kg group) showing expression range between weak positive and moderate positive of TLR4 (+, score 1) and (++, score 2). (H)(I), Group V (Ivermectin 1.5 mg/kg group) showing expression range between weak positive and moderate positive of TLR4 (+, score 1) and (++, score 2), magnification 100× and 400×, respectively. The (yellow arrow) shows TLR4 expression and the (red arrow) shows other cells (such as congested red blood cells, inflammatory lymphocytes and fibrin).

sympathetic tone, release of endothelin-1, stimulation of adhesion molecules, and a decrease in nitric oxide (NO) production. Subsequently, this condition results in thrombi formation, fibrosis, dysfunction or destruction of vascular endothelial cells, and the possibility of vasculitis progression and subsequent cardiovascular events (12,25). Moreover, this drug increases the anti-inflammatory cytokine IL-10 levels, which preserves vascular function and homeostasis, restrains the host immune response, and prevents endothelial cell damage (26). Ivermectin inhibits the development of pulmonary fibrosis by suppressing phosphorylation of signal transducer and activator of transcription 3 (STAT3) and p21-activated kinase (PAK1) pathways, which are responsible for the stimulation of TGF-β and the production of IL-6 by macrophages (27).

The results of serum CRP levels in the ivermectin groups showed a significant reduction compared to the disease group. A similar study by Ahmed et al, is concordant with this outcome; and showed that CRP levels dropped after receiving ivermectin for five days (28). Furthermore, ivermectin reduced the synthesis of CRP, an acute-phase protein in the liver. Elevated CRP levels may result in complement system activation, lymphocyte infiltration, and the production of immune cells and inflammatory cytokines in response to the sensitization by ovalbumin and LPS. Moreover, inflammatory reactions may disturb the levels of NO, formation of a blood clot, and elevated endothelin-1 concentration. Thus, ivermectin lowers its level and decreases sheer stress, damage in the vascular cells, endothelial dysfunction, and inflammation severity (28,29).

In our study, the mean serum level of MPO ANCA in the ivermectin groups was significantly reduced compared to the disease group. Ivermectin modulates innate and adaptive immune responses mediated by ovalbumin and LPS by inhibiting B cell responses to T cells and the secretion of neutrophils into the bloodstream. Consequently, the drug prevents neutrophil priming, the formation of NETs, ANCA generation, and the translocation and immunogenicity of MPO on the surface. Besides, it reduces the complement component mediated opsonization and degradation of vascular endothelial cells, reactive oxygen species formation, and interrupts the coagulation cascade and the permeability of inflammatory cells from the circulation into the vessel wall and surrounding tissue. These processes result in vasculitis progression, vascular endothelial cell dysfunction and rupture (30,31).

Conclusion

Ivermectin can modulate innate immune response by suppressing pro-inflammatory cytokines, such as IL-6 and the expression of TLR4 in response to various pathogens. The drug can maintain endothelial tissue homeostasis by decreasing ANCA binding to the surface, neutrophil priming, oxidative stress, and subsequent cell destruction.

Acknowledgments

The authors of this research would like to express gratitude to

the employees and team members of the College of Pharmacy/ Mustansiriyah University in Baghdad, Iraq for their ongoing assistance in helping and providing a suitable environment to finish this study.

Authors' contribution

Conceptualization: Nadia H Mohammed, Inam Sameh Arif.
Data curation: Teeba Sarem Sadeq, Nadia H Mohammed, Inam Sameh Arif.
Formal analysis: Teeba Sarem Sadeq, Nadia H Mohammed, Inam Sameh Arif.
Investigation: Teeba Sarem Sadeq.
Methodology: Nadia H Mohammed, Inam Sameh Arif.
Resources: Teeba Sarem Sadeq, Nadia H Mohammed, Inam Sameh Arif.
Supervision: Nadia H Mohammed, Inam Sameh Arif.
Validation: Nadia H Mohammed, Inam Sameh Arif.
Visualization: Teeba Sarem Sadeq.
Writing–original draft: Teeba Sarem Sadeq.

Conflicts of interest

The authors declare no conflict of interests.

Ethical issues

The study was conducted after consideration of the ethical issues and after obtaining permission from the scientific and animal ethics committee at the College of Pharmacy/ Mustansiriyah University, with approval number 22, Date: 10/7/2023. Guidelines regarding animal experimentation approved by the United States National Institutes of Health (NIH, 1978) have been followed. Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

Funding/Support

The authors did not receive any source of fund.

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