doi.org/10.36721/PJPS.2025.38.1.REG.249-259.1

Vitamin C inhibits LPS-induced secretion of inflammatory cytokines in peripheral blood

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Abstract: Despite extensive evidence demonstrating that vitamin C suppresses the secretion of inflammatory cytokines, research exploring its impact on cytokine release from peripheral blood cells upon bacterial antigen stimulation remains limited. This research investigated the influence of vitamin C on LPS-triggered secretion of cellular inflammatory cytokines in peripheral blood. Ten healthy volunteers provided heparinized whole blood, diluted with RPMI-1640, and distributed into containers. Cytokines (IL-10, IFN-γ, IL-4, TNF-α, IL-2, and IL-6) were quantified using flow cytometric cytokine detection kits. Results showed that LPS significantly increased IL-10 (baseline 2.7±1.35 pg/mL to 34.1±14.86 pg/mL), TNF-α (1.5±0.71 pg/mL to 1136.4±476.56 pg/mL) and IL-6 (1.8±0.59 pg/mL to 2033.4±755.29 pg/mL) levels, while IFN-γ, IL-4 and IL-2 remained unaffected. Vitamin C treatment significantly inhibited the secretion of IL-10 (23.5±5.5 pg/mL, P=0.0019), TNF-α (407.3±96.44 pg/mL, P=0.0389) and IL-6 (1572.4±444.43 pg/mL, P=0.0275). Both LPS and vitamin C caused a mild reduction in red blood cell counts, but no hemolysis was observed. These findings highlight vitamin C's potential to inhibit LPS-induced inflammation in peripheral blood, providing a basis for its therapeutic application in bacterial infection-related inflammatory diseases.

Keywords: Vitamin C, LPS, peripheral blood, inflammatory cytokines.

Submitted on 06-12-2024 – Revised on 17-12-2024 – Accepted on 08-02-2025

INTRODUCTION

Vitamin C, which is chemically known as ascorbic acid, refers to a water-soluble vitamin fundamental for individual fitness (Dosedel *et al*., 2021; Alhassan and Ahmed, 2016). Evidence suggests it influences a variety of inflammatory reactions and its role in regulating inflammatory cytokines has garnered significant research interest (Mousavi *et al*., 2019). Research investigating the impacts of vitamin C on inflammatory cytokines has gained momentum due to its potential to modulate immune responses and inflammation. A deficiency in vitamin C is linked to elevated production of pro-inflammatory cytokines, which can exacerbate inflammatory conditions (Carr and Maggini, 2017). Additionally, vitamin C levels are correlated with markers of inflammation, suggesting that sufficient intake may help reduce low-grade inflammation (Cui *et al*., 2024). Among sufferers who received total knee replacement, higher preoperative vitamin C levels were linked to lower inflammatory indicators including interleukin-6 (IL-6) and IL-1β, highlighting its potential to mitigate inflammation associated with surgical stress (Shah *et al*., 2020). Overall, vitamin C acts as a key modulator of inflammatory responses and holds promise as a therapy for the treatment of inflammatory disorders.

As a crucial element of the Gram-negative bacterial cell wall, lipopolysaccharide (LPS) comprises lipid A, core polysaccharides and O antigens (Paracini *et al*., 2022). As a potent immune stimulant, LPS can trigger the host cells to generate large quantities of inflammatory cytokines, including interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and IL-1β. It initiates multiple signaling pathways via binding to Toll-like receptor 4 (TLR4) on the surface of macrophages and other immune cells, thereby activating the immune response (Dold *et al*., 2021). This process is pivotal in infections, trauma, and other inflammatory diseases. Nonetheless, overactive inflammation may give rise to adverse outcomes, including sepsis and other related conditions.

Although there have been studies on LPS stimulation of blood cells, most of them have focused on experiments

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involving isolated individuals or a few types of blood cells (Vijayan *et al*., 2019; Sadeghi *et al*., 2020; Wang *et al*., 2020; Liddle *et al*., 2021; Farias-Jofre *et al*., 2022; Jorda *et al*., 2024). To date, there has been no systematic research on the direct use of LPS to stimulate peripheral blood and assess its inflammatory factors. A study on LPS stimulation of macrophages derived from human peripheral blood mononuclear cells revealed significant differences in metabolic responses compared to mice, particularly in glycolytic pathway activity (Vijayan *et al*., 2019). It was found that BRD4 promotes cellular senescence and inflammation in LPS-stimulated macrophages and plays a key role in lipid uptake during atherosclerosis (Wang *et al*., 2020). Another research demonstrated that daily apple consumption by obese individuals reduced the secretion of inflammatory biomarkers from peripheral blood mononuclear cells (PBMCs) under LPS stimulation. Another study found that pregnancy significantly altered the inflammatory response of PBMCs and neutrophils to LPS, indicating immune modulation during pregnancy (Farias-Jofre *et al*., 2022). Additionally, the antioxidant and anti-inflammatory effects of rosmarinic acid on peripheral blood mononuclear cells under LPS stimulation were investigated, highlighting its potential in modulating immune responses (Sadeghi *et al*., 2020). These studies reveal various effects of LPS stimulation on peripheral blood cells, including metabolic responses, inflammatory cytokine release, and immune modulation during pregnancy, providing valuable insights for understanding immune response mechanisms and developing anti-inflammatory therapies. However, these studies were all based on experimental designs where peripheral blood cells were isolated before being stimulated with LPS, and therefore, do not fully reflect the direct effects of LPS on whole blood, which contains a complex array of components.

Currently, many studies have reported that vitamin C is capable of inhibiting inflammation and oxidative stress induced by LPS (Zhang XY *et al*., 2018; Pires *et al*., 2024). For example, vitamin C alleviates myocardial injury and protects against endotoxin-induced cardiomyopathy by reducing oxidative stress, cytokine expression, and pyroptosis (Shati *et al*., 2022; Zhang P *et al*., 2022). In renal cell models, it mitigates LPS-induced HK-2 cell damage by reducing necrosis and inflammatory factor expression (Xu *et al*., 2022). Vitamin C also suppresses hyperinflammation in chronic myeloid leukemia cells and delays cellular senescence while enhancing regeneration in LPS-induced senescent hAPCs (Teawcharoensopa and Srisuwan, 2023; Pires *et al*., 2024). Furthermore, it promotes anti-inflammatory effects in microglial cells by activating the M2 phenotype (Ruggiero *et al*., 2024).

Nevertheless, it is still uncertain if vitamin C can suppress LPS-induced inflammation in whole blood, which contains a variety of blood cells and plasma components. Therefore, this study aims to explore the experimental conditions for LPS stimulation of whole blood and the inflammatory factors it induces, as well as to assess the modulatory influence of vitamin C on LPS-induced inflammatory factors. By clarifying how vitamin C suppresses the release of inflammatory factors in LPS-triggered whole blood, this study further enriches our comprehension of antioxidant function in inflammatory and immune regulation. This not only provides theoretical support for the application of vitamin C in infectious diseases, particularly bacterial infection-related inflammatory diseases but also lays the foundation for the advancement of novel adjunctive treatment approaches. In addition, this research validates the potential of vitamin C to suppress inflammatory factor secretion in whole blood, providing strong evidence for its use in adjunctive therapy for acute conditions such as acute inflammation and sepsis.

MATERIALS AND METHODS

Participants

An aggregate of 10 healthy volunteers were recruited for this study, including 5 males and 5 non-pregnant females, aged 20 to 30 years. Heparinized vacuum blood collection tubes were employed for collecting peripheral blood samples from the volunteers. The study gained approval from the Medical Ethics Committee of the First Affiliated Hospital of Guangdong Pharmaceutical University. The authorization number for medical ethics is Medical Ethics [2020] No. (86).

Reagents

Lipopolysaccharide (LPS) from E. coli O111 (Catalog No. S1732) and vitamin C (Catalog No. ST1434) were sourced from Shanghai Beyotime Biotechnology Co., Ltd, China. In this study, LPS and vitamin C were diluted using 0.9% saline. Roswell park memorial institute medium 1640 (RPMI-1640) basal cell culture medium, sterile polypropylene EP tubes, polystyrene cell culture plates and additive-free borosilicate glass blood collection tubes were acquired from Wuhan Service Biotechnology Co., Ltd, China.

Red blood cell count

Anticoagulated whole blood was placed in the Mindray BC-6900 hematology analyzer to measure various blood cell counts, including the red blood cell number.

Investigation of cell inflammatory cytokine

Cytokine levels of interferon-γ (IFN-γ), IL-4, IL-2, IL-6, TNF-α, and interleukin-10 (IL-10) were assessed via the human Th1/Th2 cytokine detection kit (Flow Cytometry Fluorescence Method, Catalog No. P110600103), purchased from Jiangxi Cell Gene Biotechnology Co., Ltd, China. Plasma samples were analyzed for these six cytokines using a BD flow cytometer and the provided assay kit.

LPS Stimulation of Whole Blood

Fresh peripheral blood or whole blood diluted with RPMI-1640 was placed into heparinized glass tubes, with the time point set to 0 hours. Blood cell counts were performed using the Mindray BC-6900 hematology analyzer. One blood sample was reserved, centrifuged to obtain the plasma, and stored in a deep freezer for future use or directly applied to cytokine detection. Heparinized whole blood was then transferred into several containers, each containing 500μL. Depending on the experimental requirements, LPS (final concentration of 10ng/mL) or vitamin C (50μM) was added to the containers, which were then incubated at 37°C. After 2 hours of incubation, a blood cell count was performed, and the whole blood was also centrifuged to collect the plasma for subsequent cytokine analysis. Each time, 500μL of whole blood from one container was used per test and the blood was not reused.

STATISTICAL ANALYSIS

SPSS 27.0 statistical software was utilized for analyzing the data. The data were presented as mean ± standard deviation. Paired t-tests were adopted for comparing disparities across two categories and assessing the statistical significance of group differences. The significance threshold of $P<0.05$ was adopted for hypothesis testing. A P-value below 0.05 was of statistical significance.

RESULTS

Polystyrene and Polypropylene Materials, and RPMI-1640 Significantly Stimulate Nonspecific Inflammatory Cytokine Secretion in peripheral blood

Experimental conditions significantly influenced cytokine levels in whole blood. In the borosilicate glass tube group, IL-6 and TNF-α levels remained below the upper reference limits (fig. 1, fig. 2A, B). In contrast, groups using EP tubes, PE plates, or RPMI-1640 medium showed significantly elevated levels. For example, IL-6 levels reached 1000pg/mL in the EP+RPMI-1640 group, compared to less than 10pg/mL in the glass tube group ($p<0.0001$, fig. 2A). Similarly, TNF-α levels exceeded 1000pg/mL in the PE+RPMI-1640 group, significantly higher than in the glass tube group ($p<0.0001$, fig. 2B). These findings indicate that borosilicate glass tubes significantly reduce nonspecific cytokine stimulation, while RPMI-1640 medium and polymer materials notably enhance nonspecific inflammatory responses.

LPS Stimulation of peripheral blood primarily induces tnf-α, il-10, and il-6 secretion

The use of borosilicate glass tubes effectively minimized nonspecific cytokine secretion in heparinized whole blood. Following LPS stimulation (10ng/mL) for 2 hours, levels of TNF-α, IL-10 and IL-6 were significantly elevated compared to the control group (fig. 3A-C). TNF-α, IL-10, and IL-6 exceeded the upper reference limits and were markedly higher than other cytokines analyzed. For example, TNF-α levels increased from less than 10 pg/mL in the control group to over 1000pg/mL in the LPS-treated group ($p<0.0001$, fig. 3C). Similarly, IL-10 and IL-6 levels also showed significant increases ($p< 0.0001$, fig. 3A, B). These findings confirm the pronounced inflammatory response induced by LPS and highlight the importance of minimizing nonspecific stimulation during experimental setups

Vitamin C Inhibits LPS-triggered TNF-α, IL-10 and IL-6 Secretion

Vitamin C (50μM) significantly suppressed LPS-induced cytokine secretion in borosilicate glass tubes. Compared to the LPS group, TNF-α, IL-6, and IL-10 levels were significantly reduced in the LPS +Vitamin C group ($p<0.05$, fig. 4A-C), with TNF-α decreasing from approximately 600pg/mL to 400pg/mL and IL-6 from 2500pg/mL to 1500pg/mL. These results indicate that vitamin C effectively alleviates LPS-induced inflammatory responses.

LPS and Vitamin C Cause a Mild Decrease in Red Blood Cells But Do Not Reach Hemolysis Threshold

The hemolysis percentage was calculated via contrasting the red blood cell count in the LPS or vitamin C-treated groups with that in the untreated control category. The outcome demonstrated that after adding vitamin C, the hemolysis of red blood cells (RBC) was slightly increased in the presence of LPS, but it did not reach the hemolysis threshold (fig. 5).

DISCUSSION

This research reexamined experimental conditions for *in vitro* LPS stimulation of peripheral blood. Although many studies have used RPMI-1640 cell culture medium to dilute whole blood for subsequent experiments (Rodas *et al*., 2021; Hass *et al*., 2023; Koceniak *et al*., 2023), our findings indicate that the commonly used RPMI-1640 medium can stimulate the nonspecific secretion of inflammatory cytokines in whole blood. This phenomenon may be related to the composition of the medium and the stimulation duration. Components in the medium, such as glucose and amino acids, are essential nutrients for cell growth; however, their metabolic byproducts, such as lactate and ammonia, may adversely affect cellular functions. For instance, the accumulation of lactate may lead to a decrease in the pH of the medium, thereby inhibiting normal cell growth and function, while the accumulation of ammonia may exhibit toxicity and induce inflammatory responses. Moreover, replacing the medium may remove cytokines, extracellular vesicles, and other signaling molecules secreted by the cells, thereby disrupting intercellular communication.

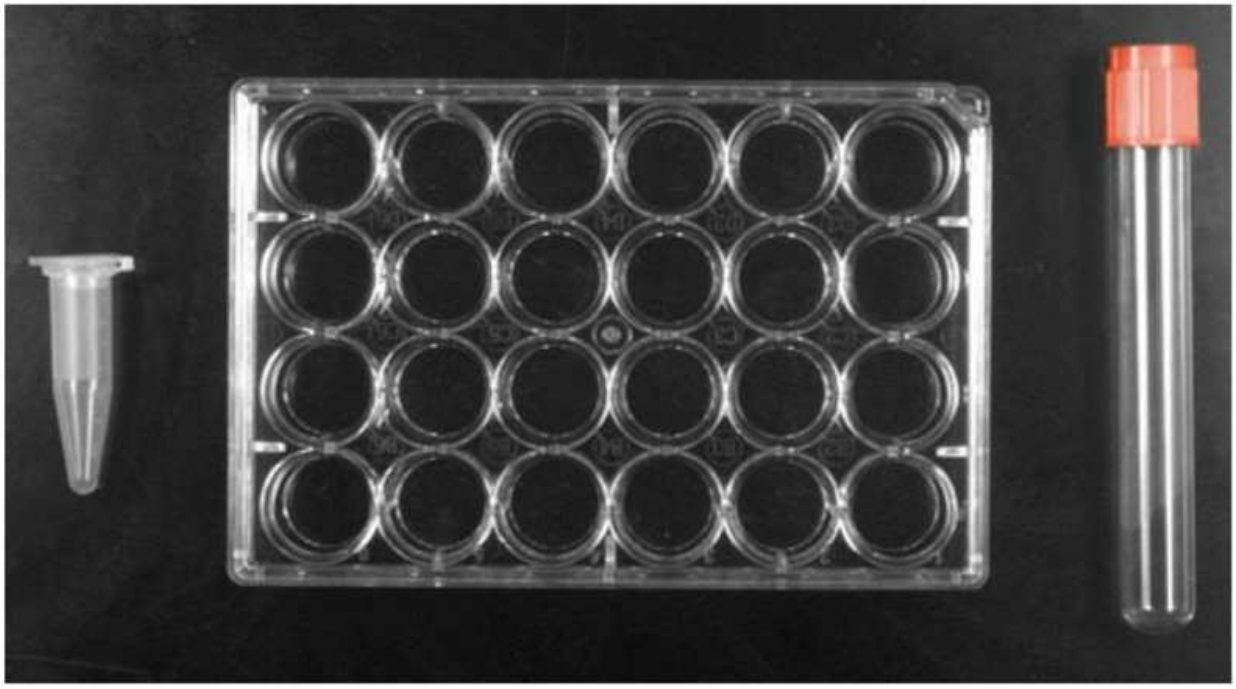

Fig. 1: Photographs of three different types of containers. From left to right: polypropylene EP tube, polyethylene 24-well cell culture plate, and borosilicate glass tube.

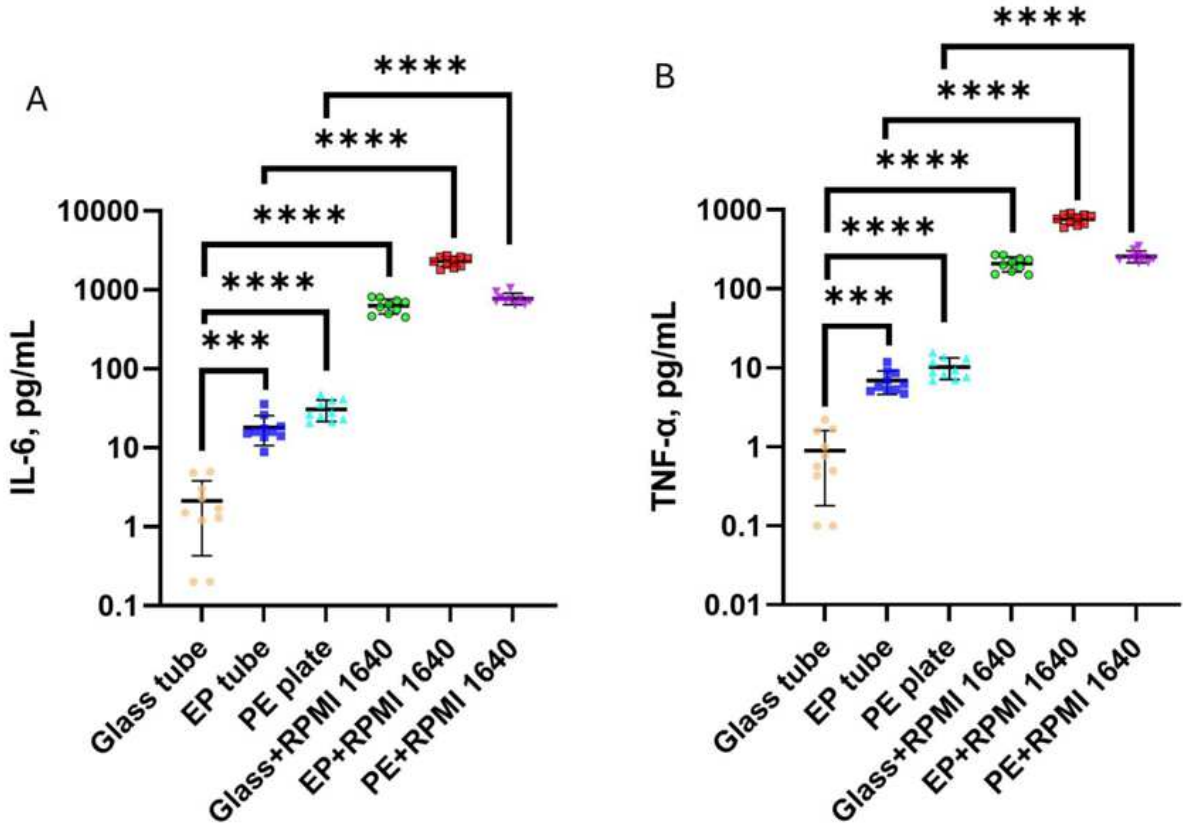

Fig. 2: Inflammatory cytokine levels in peripheral blood cells under different conditions. (A-B) The graphs show IL-6 and TNF-α cytokine levels in fresh heparinized whole blood after 2 hours of incubation at 37°C under different conditions. EP, polypropylene EP tube; PE, polyethylene plate for cell culture (***P<0.001; ****P<0.0001).

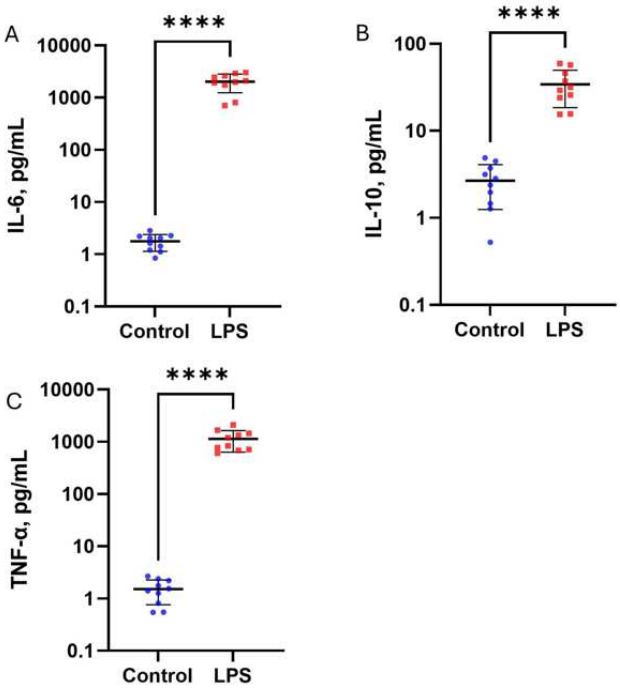

Fig. 3: Changes in cytokine levels after LPS induction of whole blood. The heparinized whole blood was incubated at 37°C after stimulation with LPS in borosilicate glass tubes. The levels of IL-6 (A), IL-10 (B), and TNF-α (C) were assessed following 2-hour incubation. The changes in cytokine levels are shown (****P< 0.0001).

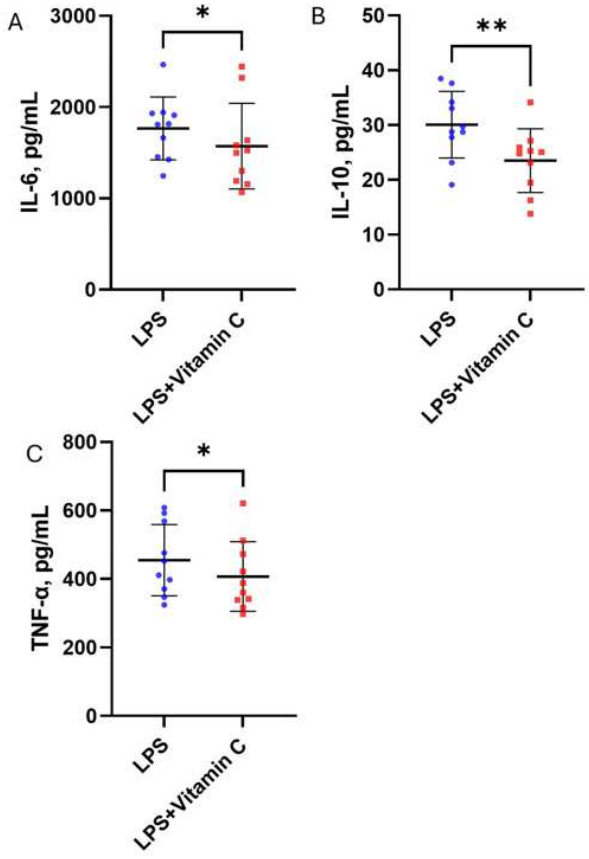

Fig. 4: Impact of vitamin C on LPS-induced cytokine levels in peripheral blood. The levels of IL-6 (A), IL-10 (B), and TNF-α (C) were measured (*P<0.05; **P<0.01).

This disruption in communication may prompt the cells to accelerate the secretion of signaling molecules within a short time to re-establish communication, potentially further enhancing the secretion of inflammatory cytokines (Vis *et al*., 2020).

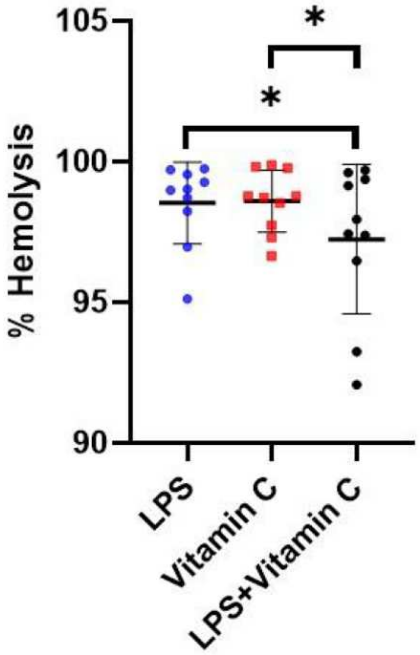

Fig. 5: Percentage of Red Blood Cell Hemolysis after 2 Hours of LPS and Vitamin C Treatment in Heparinized Whole Blood. Red blood cell counts for each group were measured using a hematology analyzer, and the hemolysis rate for each group was calculated by comparing the red blood cell count with the average count of the untreated control group (without vitamin C and LPS; **P*<0.05).

In our experiment, the stimulation time was relatively short (less than 4 hours), whereas many studies used stimulation times exceeding 20 hours. Prolonged storage of whole blood could lead to a decrease in cytokine levels in the supernatant (Liu C *et al*., 2021). However, we believe that the impact of early nonspecific stimulation should not be overlooked in experiments. Therefore, we recommend using whole blood directly, rather than diluted blood, to avoid the potential stimulating effect of RPMI-1640 on blood cells when performing *in vitro* LPS stimulation and measuring inflammatory cytokines including TNF-α and IL-6. Also, this research evaluated the influences of diverse container materials on peripheral blood and found that polypropylene EP tubes and polystyrene cell culture plates can stimulate peripheral blood cells to secrete inflammatory cytokines (fig. 2). While previous experiments have used polystyrene cell culture plates or dishes for bacterial antigen stimulation of blood cells (Liu Y *et al*., 2018; Nesseler *et al*., 2019; Rodas *et al*., 2021; Hass *et al*., 2023) and cytokine detection, in this study still recommend using borosilicate glass tubes for peripheral blood cell stimulation experiments. Polypropylene EP tubes and polystyrene materials may affect blood cell activation (Hwang *et al*., 2019; Cobanoglu *et al*., 2021; Kik *et al*., 2021; Dievernich *et al*., 2022).

For example, the toxic effects of polypropylene microplastics on peripheral blood lymphocytes were studied, revealing their potential to induce oxidative stress and cytotoxicity (Hwang *et al*., 2019); The immune response triggered by polypropylene mesh used in abdominal wall hernia repair was examined, revealing its potential to activate peripheral blood monocytes and elicit reactions from various immune cells (Dievernich *et al*., 2022). Unfunctionalized polystyrene nanoparticles were found to induce genotoxicity and pro-inflammatory responses in human peripheral blood monocytes (Kik *et al*., 2021). The impact of polystyrene microplastics on peripheral blood lymphocytes was assessed using genotoxicity markers, revealing their potential to cause DNA damage and cytotoxicity (Cobanoglu *et al*., 2021). Borosilicate glass tubes effectively reduce the nonspecific stimulation of inflammatory cytokine secretion in peripheral whole blood through their chemical stability, surface inertness, low protein adsorption, and smooth surface properties (Wolf *et al*., 2021). Their chemical stability and inertness prevent nonspecific reactions between blood components (e.g., proteins and blood cells) and the material surface, thereby reducing the likelihood of cell activation. The low protein adsorption of borosilicate glass helps maintain the native state of serum proteins and cytokines, avoiding immune cell activation caused by protein denaturation or excessive adsorption. The smooth surface minimizes mechanical stimulation of blood cells, reducing physical activation (Borciani *et al*., 2022) Additionally, the low thrombogenicity of borosilicate glass ensures it does not significantly activate coagulation pathways (e.g., thrombin generation and platelet activation), thereby indirectly lowering immune and inflammatory responses. Although borosilicate glass tubes may induce a certain degree of stimulation in peripheral blood cells, their nonspecific stimulation is significantly lower compared to polypropylene and polystyrene tubes, further highlighting their superiority in minimizing inflammatory cytokine secretion. Moreover, the cryopreservation process may impact the viability and functionality of isolated blood cells, potentially altering their responses and affecting their overall performance (Jerram *et al*., 2021). Therefore, in future inflammation-related peripheral blood stimulation experiments, we recommend using glass tubes and minimizing experimental procedures.

Previous research has primarily investigated the oxidative or inflammatory responses of specific blood cells stimulated by LPS. However, peripheral blood is complex, consisting mainly of plasma and blood cells, including white blood cells, RBC, and platelets (Hoffman *et al*., 2022). Limited attention has been given to

assessing the impacts of LPS stimulation on whole peripheral blood. Our study investigated the secretion of inflammatory cytokines following LPS induction of peripheral blood to evaluate the impact of LPS on cytokine secretion in whole blood containing these complex components. The outcomes demonstrate that in *ex vivo* experiments, stimulation of LPS triggered the secretion of TNF-α, IL-10, and IL-6 in peripheral blood (fig. 3). Monocytes, as well as macrophages, are the primary cells responsible for IL-6 secretion in peripheral blood. When stimulated by inflammatory factors (such as LPS) or inflammatory cytokines, these cells rapidly release IL-6 to initiate the inflammatory response (Shimoda *et al*., 2023).

Macrophages and monocytes also represent the main sources of TNF-α in peripheral blood. Upon stimulation by LPS or inflammatory factors, quickly release TNF-α to initiate and regulate the inflammatory response (Ollendorf *et al*., 2011; Yin *et al*., 2022). Although neutrophils are not the crucial source of IL-6, they have the capacity to secrete small quantities of IL-6 upon bacterial or inflammatory stimuli, thereby enhancing the local inflammatory response (Wypych and Marsland, 2018). Similarly, while neutrophils are not the main source of TNF-α, under certain inflammatory conditions such as severe infection or systemic inflammatory response, they can release small amounts of TNF-α to enhance the local inflammatory response, supporting the activation and recruitment of other immune cells (Scapini and Cassatella, 2014). Some activated T cells (e.g. helper T cells) can also produce IL-6 during immune responses, particularly in regulating B cell differentiation and antibody generation (Scheller *et al*., 2011). Therefore, the increased levels of TNF-α, IL-10, as well as IL-6, following LPS induction of peripheral blood may result from the secretion and interactions of multiple cell types.

This study found that vitamin C significantly reduced LPS-induced IL-10, TNF-α, and IL-6 levels in peripheral blood compared to LPS treatment alone (fig. 4). Previous studies suggest that vitamin C regulates inflammatory responses by targeting key signaling pathways, such as MAPK and NF-κB, which are critical in LPS-induced cytokine production. Vitamin C may reduce phosphorylation of p38, JNK, and ERK1/2 in the MAPK pathway, suppressing their activation and lowering pro-inflammatory cytokines like TNF-α, IL-6 and IL-1β. It may also inhibit IκBα degradation, blocking NF-κB activation and reducing inflammation-related gene expression.

Additionally, vitamin C's antioxidant properties help mitigate LPS-induced ROS production, which amplifies inflammatory signals, further reducing cytokine secretion. While these mechanisms provide insights into its anti-inflammatory effects, the precise molecular pathways in peripheral blood remain unclear. Future research should focus on validating its role in signaling pathways like NF-κB and MAPK and explore gene expression changes to better understand its mechanisms in peripheral blood inflammation. This would strengthen the theoretical basis for its therapeutic use in inflammation-related diseases. Several other natural substances or compounds have also been reported to inhibit LPS-induced cytokine secretion (Bian *et al*., 2019; Cao *et al*., 2019; Hung *et al*., 2019; Zhang J *et al*., 2019; Zhao *et al*., 2019; Wu *et al*., 2020; Pitchakarn *et al*., 2024). For example, nattokinase reduces the generation of pro-inflammatory cytokines via inhibiting the LPS-mediated TLR4 as well as NOX2 signaling pathways, thereby alleviating inflammation (Wu *et al*., 2020); caffeine decreases LPS-triggered IL-1β and IL-18 secretion via suppressing the MAPK/NF-κB pathway (Zhao *et al*., 2019); bavachin reduces the release of IL-6 and IL-12 induced by LPS via the suppression of NLRP3 inflammasome activation (Hung *et al*., 2019); kaempferol inhibits LPS-triggered IL-1β and TNF-α generation, thereby mitigating the inflammatory response (Bian *et al*., 2019); punicalagin reduces the secretion of IL-6 and TNF-α in LPS-triggered RAW264.7 macrophages via the suppression of the FoxO3a/autophagy signaling pathway (Cao *et al*., 2019); curcumin decreases LPS-induced neuroinflammation and pro-inflammatory cytokine secretion via the TREM2/TLR4/NF-κB pathway (Zhang J *et al*., 2019). Interestingly, similar to vitamin C, soybean oligopeptide treatment also induces mild hemolysis but significantly reduces inflammatory cytokine secretion (Pitchakarn *et al*., 2024). These compounds have demonstrated the ability to inhibit LPS-triggered pro-inflammatory cytokines in numerous *in vitro* or *in vivo* models, as well as alleviate inflammation through different signaling pathways, showing potential for use in treating inflammatory diseases.

Mild hemolysis in the RBC of the LPS and vitamin C-treated groups was found in this study (fig. 5). The primary target of LPS is immune system cells, particularly monocytes and macrophages, through which it induces the generation of inflammatory cytokines and triggers an inflammatory response. The release of numerous inflammatory cytokines triggered by LPS may affect the stability of the red blood cell membrane (Bozza and Jeney, 2020), leading to hemolysis. In other experiment, with the relatively low concentration of vitamin C, mild hemolysis was also observed, consistent with previous reports (Dosedel *et al*., 2021). As an antioxidant, vitamin C exerts a protective effect on RBC under oxidative stress, reducing hemolysis and membrane damage. However, at lower concentrations, vitamin C may contribute to increased hemolysis, while higher concentrations exert a protective effect against hemolysis (Dosedel *et al*., 2021). Low concentrations of vitamin C may also influence the stability of the red blood cell membrane through other mechanisms, resulting in cell

rupture. During hemolysis, hemoglobin and heme released as erythrocyte-derived danger-associated molecular patterns (eDAMPs) can activate the TLR4 signaling pathway and NF-κB, thereby promoting the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. Additionally, free hemoglobin and heme catalyze the production of reactive oxygen species (ROS) through the Fenton reaction. ROS can further activate NF-κB and upregulate adhesion molecules such as VCAM-1 and ICAM-1, amplifying the inflammatory response. Hemolysis also releases erythrocyte-derived microparticles (MPs), which carry inflammatory mediators and further enhance cytokine secretion (Nader *et al*., 2020). Even mild hemolysis may interfere with cytokine level measurements through these mechanisms, and its potential impact should be carefully considered in experimental design.

This study has several limitations that should be addressed in future research. First, the sample size of 10 healthy volunteers, while reasonable for a preliminary investigation, limits the statistical power and generalizability of the findings. Future studies with larger and more diverse sample sizes will be essential to validate these results and ensure their applicability to broader populations, including those with specific health conditions or diseases involving inflammatory responses.

Second, this study focused on six inflammatory cytokines, excluding others such as IL-1β and IL-8, which play critical roles in the inflammatory cascade. While these six cytokines provide important insights, the inclusion of additional markers in future investigations could offer a more comprehensive understanding of how vitamin C modulates the immune response to LPS stimulation. This would help to delineate the broader immunomodulatory effects of vitamin C on both pro-inflammatory and anti-inflammatory pathways.

Lastly, the findings are based on an ex vivo model, which, while valuable for controlled experimental conditions, may not fully replicate the complexities of *in vivo* environments. Factors such as metabolism, systemic immune interactions and tissue-specific responses may alter the observed effects of vitamin C. To address this, future studies should prioritize validating these findings in animal models and clinical trials. For instance, using rodent models of sepsis or inflammation could help evaluate the effects of vitamin C *in vivo* under controlled but physiologically relevant conditions. Additionally, small-scale clinical studies in human patients suffering from inflammatory diseases could explore the therapeutic potential of vitamin C and assess its efficacy and safety in real-world settings. These steps will be critical to understanding the translational relevance of the findings and advancing vitamin C as a potential treatment for inflammation-related conditions.

CONCLUSION

This research evaluated the impacts of LPS on the secretion of inflammatory cytokines in peripheral blood and verified the inhibitory effects of vitamin C. The outcomes demonstrated that borosilicate glass tubes rather than polypropylene or polystyrene tubes could minimize the non-specific stimulation of the release of inflammatory cytokine in peripheral whole blood. The RPMI-1640 medium commonly used in cell culture also significantly non-specifically stimulates the peripheral whole blood to secrete inflammatory cytokines. LPS notably triggered the release of IL-10, TNF-α and IL-6 in peripheral whole blood, which was elevated beyond clinical reference values, while no notable alterations were detected in the secretion of IFN-γ, IL-4 and IL-2. Under vitamin C treatment, the release levels of these inflammatory cytokines were notably reduced, demonstrating the potential application of vitamin C in inhibiting inflammation caused by bacterial infections. Additionally, the administration of vitamin C and LPS slightly decreased red blood cell count but did not reach hemolysis levels, indicating that vitamin C has good safety under the conditions of this experiment.

ACKNOWLEDGMENTS

We extend our gratitude to Dr. Yue Wu, a student at Guangdong Pharmaceutical University, for her technical assistance. This research was funded by the Science and Technology Program of Guangzhou (grant No. 202103000051).

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