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Original Article

The effects of glucocorticoid plus intravenous immunoglobulin (IVIG) vs IVIG alone on platelet activation in children with Kawasaki disease

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ABSTRACT

Background. Even though intravenous immunoglobulin (IVIG) is a current treatment for Kawasaki disease (KD), 10–20% of patients require additional therapy. This study seeks to investigate the therapeutic effects of glucocorticoids plus IVIG on KD and to ascertain the subsequent effect on platelet activation during the acute phase.

Methods. A total of 32 children with KD were randomly classified into two groups: the experimental group (16 cases) and the control group (16 cases). The control group was exposed to IVIG (2 g/kg), whereas children in the experimental group were treated with IVIG (2 g/kg) + glucocorticoid. Peripheral venous blood samples were obtained from all participants before treatment as well as three days post-treatment to test platelet activation levels with procaspase activating compound-1 (PAC-1) antibody, Toll-like receptor 4 (TLR4), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), procalcitonin (PCT), and C-reactive protein (CRP). Fever duration post-treatment was documented for both groups. Additionally, the coronary arteries in both groups were evaluated during three months of treatment.

Results. After treatment, the experimental group had remarkably lower levels of TNF-α, CRP, PCT, IL-6, PAC-1, and TLR4 relative to the control group. The fever persistence rate was considerably elevated in the control group compared to the experimental group (log-rank, P=0.024). In addition, the z-score of coronary artery size dropped after IVIG + glucocorticoids treatment compared to the control group, although this difference was not significant.

Conclusions. The IVIG + glucocorticoids can quickly mitigate the inflammatory response and platelet activation. Moreover, it can also improve clinical symptoms in children with KD.

Key words: Kawasaki disease, corticosteroid, intravenous immunoglobulin, platelet activation, coronary artery lesions.

Kawasaki disease (KD) is an acute self-limiting inflammatory disorder related to systemic vasculitis.[1] Coronary vessel wall inflammation in KD can cause coronary artery abnormalities (CAAs).[1] Moreover, coronary vascular endothelium damage can potentially activate platelets, thereby initiating a cascade of further vascular damage.[2-4] Literature studies depict that platelet activation and systemic inflammatory changes during the acute phase of KD support the release of procaspase activating compound-1(PAC-1) and Toll-like receptor 4 (TLR4), thereby accelerating disease progression.[5,6] Hence it provides a theoretical basis for the treatment of KD with an antagonist that modulates platelet-activating factors and suppresses inflammatory response.

Exposure to intravenous immunoglobulin (IVIG) reduces the incidence of coronary aneurysms from 25% to only nearly 9%.[7] Even with timely IVIG treatment, around 4% of affected children

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progress to coronary anomalies, and 1% can even develop giant aneurysms.[8] A recrudescent or persistent fever following infusion with IVIG is one of the most powerful risk factors for a coronary aneurysm - IVIG resistance.[9] About 15-20% of patients develop IVIG resistance at the end of IVIG therapy and are considered more susceptible to developing CAAs. IVIG resistance rates have consistently increased from about 7% in 2003 to ~23% in 2014, with a concomitant elevation in aneurysms of the coronary artery.[10] Several treatment strategies have been tested to further decrease the risk of coronary artery aneurysms.[11-13] The addition of steroids to conventional treatment is one option; however, the effect remains controversial. A potential positive effect of glucocorticoids in the acute phase of KD has been suggested in recent studies.[13-16] This study aimed to demonstrate the impact of glucocorticoids plus IVIG in KD treatment and characterize its potential mechanisms in the acute phase of the disease.

Material and Methods

Study design

Inclusion criteria: 1) All patients meeting the KD diagnostic criteria defined by the American Heart Association (AHA) in 2017[1] : a) fever ≥5 days, b) conjunctival hyperemia in both eyes, c) oral changes, d) erythema multiforme and rashes, e) hand and foot sclerosis or peeling, f) acute non-suppurative lymphadenitis in the neck. KD was diagnosed in the presence of at least 5 of the six principal symptoms. 2) Patients without KD-specific treatment prior to admission. 3) Individuals with family members willing and able to cooperate for study completion. 4) Individuals who granted written informed consent via forms.

Exclusion criteria: 1) Individuals with concomitant severe congenital or organic diseases of the liver, heart, or kidney. 2) Individuals with a history of KD. 3) Individuals presenting symptoms of coronary artery abnormalities prior to admission. 4) Individuals who had received intravenous immunoglobulin or steroids prior to admission. 5) Individuals who took related drugs like immunosuppressants, which might influence the study.

Randomization and blinding

The randomization technique employed in this study involved a sealed envelope system. Informed consent was obtained from the parents of children who met the inclusion criteria, and treatments were randomly assigned to sealed envelopes. Following this, a physician opened a random envelope and selected the assigned treatment regimen. Patients and clinicians were not blinded to the assignment.

Study population

A total of 32 children diagnosed with KD were enrolled in the study at the Provincial Children's Hospital Affiliated with Anhui Medical University, from January 1, 2020, to December 31, 2021, with 16 cases allocated to each group. Patient baseline characteristics are shown in Table I. The Institutional Ethics Committee of the Provincial Children's Hospital, Anhui Medical University approved the study. Moreover, informed, signed consent was obtained from all involved.

Treatment groups: Individuals in the control group were administered IVIG and aspirin. The exact treatment regimen included immunoglobulin 2g/kg, intravenous drip, and single-dose application. Simultaneously, aspirin was orally administered at 30-50 mg/kg/day, gradually declining to 3-5 mg/kg/day once the fever subsided for at least three consecutive days. These conditions were maintained for two months. On the other hand, individuals in the experimental group were exposed to glucocorticoids in addition to IVIG and aspirin, as above. The glucocorticoids scheme was intravenous methylprednisolone at a dose of 2 mg/kg twice daily for three days. After a 3-day fever-free period, the oral prednisolone dose

Table I. Clinical and laboratory characteristics of experimental and control participants

Characteristics	Experimental group (n=16)	Control group (n=16)	P-value
Age (months)	32.0 (20.3-45.0)	27.0 (13.0-36.0)	0.509
Male/female	9/7	11/5	0.716
Weight (kg)	12.0 (9.6-15.6)	12.0 (9.5-14.0)	0.721
Days of illness at treatment (day)	5.1±1.9	5.1±1.9	0.927
Hemoglobin(g/dl)	10.52±1.27	11.30±1..27	0.092
White-cell count ($\times10^3/\mu L$)	13.0 (10.3-15.5)	15.2 (11.3-17.2)	0.122
Platelet count ($\times10^4/\mu L$)	312.1±127.3	375.7±145.3	0.198
D-dimer(mg/L)	1.5 (1.1-2.1)	1.1 (1.0-2.8)	0.228
CD64 (%)	5.8 (4.8-8.2)	6.1 (5.5-7.9)	0.427

was changed to 2 mg/kg. Prednisolone was also gradually reduced in 5-day steps over 15 days from 2 mg/kg/day to 1 mg/kg/day to 0.5 mg/kg/day. The total course of steroids was 18 days.

The discharge summary of the patients included medication instructions, the need for regular follow-ups, and regular notifications at each point of the assessment. Furthermore, the patients were consistently reminded to undergo relevant examinations at every assessment. At the end of each follow-up, the physician also ensured that the patient was informed about the schedule and contents for the next visit.

Sample collection

Clinical data, including age, height, weight, gender, and time of body temperature drop after treatment, were obtained. Clinical inflammatory markers procalcitonin (PCT), C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), were obtained from patients before treatment as well as three days post-treatment. The assessments of coronary arteries were carried out by measuring the luminal diameter of the main right and left coronary arteries, the left anterior descending artery, and the left circumflex artery before treatment, 2-, 4-, and 12- weeks after treatment.

Platelet activation detection by flow cytometry

Blood samples were obtained from the patients before IVIG and three days after IVIG. A 2 ml blood draw was acquired from patients and controls using a 5 ml needle and immediately transferred to plastic tubes containing EDTA. This was done to avoid manual activation of the platelets during sample collection. Additionally, during transport from the clinic to the laboratory, samples were kept at room temperature to eliminate any effect of temperature fluctuations on platelet activation. These samples were then centrifuged for 10 min. at 800 X g to obtain platelet-rich plasma (PRP). This step was followed by immediate freezing at -80°C until further analysis.

All samples were immobilized with 1% paraformaldehyde and analyzed on a FACSCanto II flow cytometer (Becton Dickinson, USA). Reagents like mouse anti-human CD61 antibody conjugated to BV510, mouse anti-human TLR4 antibody conjugated to phycoerythrin (PE), and mouse anti-human PAC-1 antibody conjugated to APC were purchased from B.D. Pharmingen (USA). As for the controls, immunoglobulins from the same mouse species were used (B.D. Pharmingen, USA) for flow cytometry. Furthermore, the CD61 marker served as an activation-independent marker of platelets. The percentage of platelets expressing TLR4 or PAC-1 was considered the fraction exhibiting specific binding (TLR4 or PAC-1 positive) minus non-specific binding (the percentage with IgG-PE conjugate) of the 10,000 platelets analyzed. Moreover, the TLR4 or PAC-1 expression assays were performed in duplicates for each blood sample, and the means were recorded.

Endpoints

The primary endpoint: levels of PAC-1 and TLR4 before treatment as well as three days after treatment.

Secondary endpoints: 1) z-scores of coronary arteries, 2) duration of fever post-treatment, 3) levels of TNF-α, IL-6, CRP, and PCT at three days post-treatment, 4) incidence of side effects during the treatment.

Outcomes

The primary outcome: changes in platelet activation levels at three days following treatment.

Secondary outcomes: 1) duration of fever (hours): from completion of initial IVIG infusion to afebrile condition, 2) changes of z-scores of coronary artery throughout the study period, 3) changes in TNF-α, IL-6, CRP, and PCT levels at three days post-treatment, 4) Frequency of all side effects during the treatment.

Statistical analysis

IBM SPSS version 22.0 (USA) aided the statistical analyses. For continuous variables, the data were depicted as the mean plus or minus standard deviations or as the median and interquartile range for normal and non-parametric data, respectively. On the other hand, categorical variables were expressed as frequencies and proportions. Data were compared by means of the Student's t-test and repetitive measure analysis of variance for normally distributed continuous variables. In contrast, Fisher's exact test was employed for categorical variables.

Furthermore, non-parametric continuous variables were assessed by means of the Mann-Whitney U test or the Wilcoxon signed-rank test. Following this, sequential persistence curves were computed by applying the Kaplan-Meier method and were subsequently compared using the log-rank test. All tests were two-tailed, and a P-value of <0.05 was considered significant.

Results

Changes in inflammatory factors

Alterations in inflammatory factors both prior to treatment and post-treatment between the two groups are depicted in Table II, which demonstrates that TNF-α, CRP, IL-6, and PCT were remarkably elevated before treatment in the two groups. However, the difference was not significant (P>0.05). Moreover, the above-mentioned markers declined at three days post-treatment, with a statistically significant difference (P<0.05). The experimental group had remarkably lower levels of TNF-α, CRP, PCT, and IL-6 relative to the control group (p<0.05).

Table II. Analysis of changes in inflammatory factors before and after treatment according to treatment groups

Variables		Experimental group (n=16)	Control group (n=16)	P
CRP (mg/L)	Before treatment	71.49 (42.1,119.38)	64.15 (51.91,85.38)	0.534
	3d after treatment	4.83 (0.5,7.84)	7.64 (4.28,11.98)	0.047
	P	<0.001	<0.001	
PCT (ng/ml)	Before treatment	0.79 (0.28,4.63)	0.25 (0.16,1.45)	0.250
	3d after treatment	0.05 (0.04,0.09)	0.16 (0.06,0.38)	0.021
	P	0.001	0.026	
IL-6 (ng/L)	Before treatment	158.38 (39.04,544.08)	86.45 (41.80,263.35)	0.059
	3d after treatment	2.00 (1.50,3.04)	3.36 (2.24,8.65)	0.023
	P	<0.001	<0.001	
TNF-α (ng/L)	Before treatment	18.75 (12.70,29.13)	17.05 (10.73,22.31)	0.402
	3d after treatment	6.20 (4.00,11.53)	12.15 (9.28,16.50)	0.020
	P	0.001	0.031	

Expression levels of PAC-1 and TLR4

Expression levels of platelet activation in the two groups are depicted in Fig. 1. The PAC-1 and TLR4 antibody targeting GPIIb/IIIa levels in both groups were remarkably elevated prior to treatment, and the difference between them was not significant (P>0.05). The two indicators declined post-treatment, with a statistically significant drop from before treatment (P<0.05). Moreover, the levels of TLR4 and PAC-1 in the experimental group were significantly lower than those in the control group at three days post-treatment (P<0.05).

Time-dependent change of the fever in patients with and without glucocorticoid therapy

Duration of fever after treatment between the two groups was determined. The Kaplan-Meier curves for the rate of cumulative persistence of fever are illustrated in Fig. 2. The 5-, 10-, 20-, 30-, 60-, and 70-hour rates of cumulative persistence of the fever were 31.3%, 6.3%, 6.3%, 0%, 0%, and 0%, respectively in the experimental group. On the contrary, the observed percentages were 50%, 25%, 12.5%, 6.3%, 6.3%, and 0%, respectively, in the control group. Furthermore, the rate of fever persistence was significantly elevated in the control group relative to the experimental group (log-rank, P=0.024). The median fever duration regression in the experimental group was 3.0 hours, whereas it was 4.5 hours for the control group.

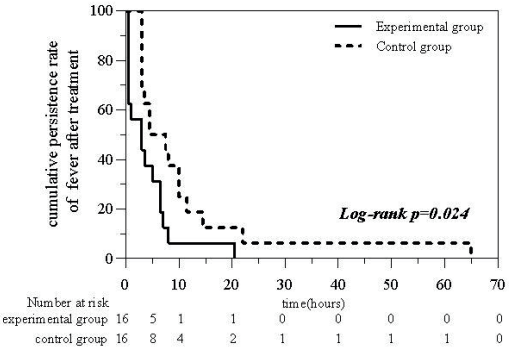

Fig. 2. Kaplan-Meier analyses for the cumulative persistence rate of fever after treatment.

The change in coronary artery z-scores over time

The change in coronary artery z-score prior to treatment relative to post-treatment between the two groups is illustrated in Fig. 3. The z-score declined sharply following IVIG + glucocorticoid therapy relative to the control group. However, the difference was not significant (P>0.05).

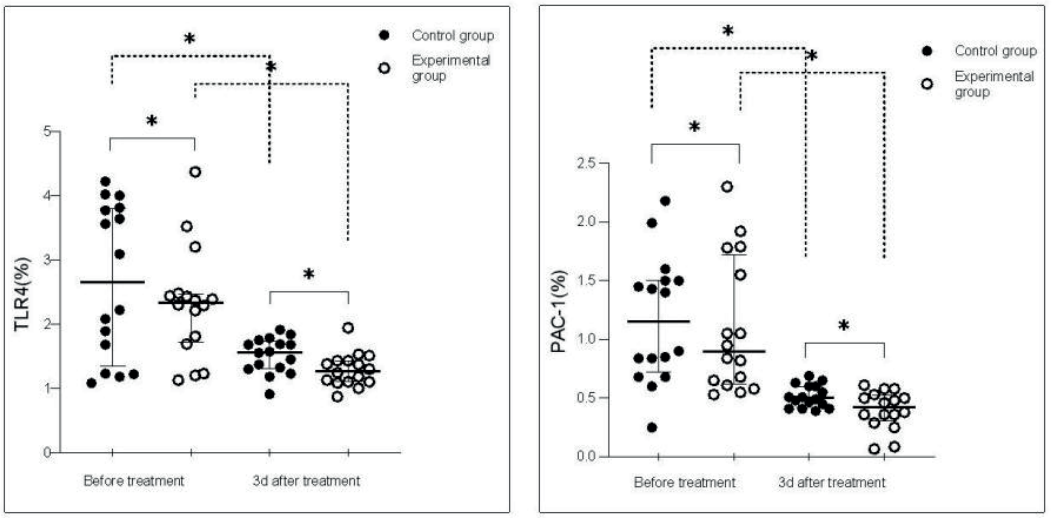

Fig. 1. Scatterplots of TLR4 (a), and PAC-1(b). Values depict the median and interquartile range of individuals in the two groups. * P<0.05

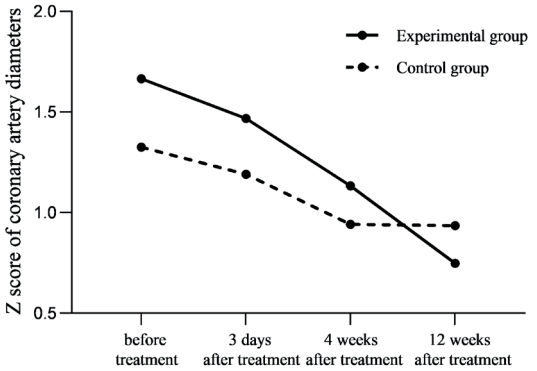

Fig. 3. Mean z-score levels of coronary artery diameters over time.

During the treatment, no significant side effects or adverse reactions to the drug were observed in any of the study participants.

Discussion

Coronary artery lesions (CALs) are among the most significant KD-related complications as well as a major determinant of long-term prognosis.[14] CALs can cause aneurysms of the coronary artery, myocardial ischemia, occlusions, myocardial infarction, or even death.[17] The vascular injury mechanism in children with KD is hypothesized to be linked to the activation of the monocyte/macrophage system *in vivo*. This releases several inflammatory mediators and factors, thereby causing vascular endothelial injury. Additionally, several studies establish that IL-2, 4, 6, 8, and 17 are positively correlated with the inflammatory response in the acute phase of KD. IL-6 even contributes to CAAs formation and vascular endothelial damage.[18-20] In this experiment, IL-6, TNF-α, PCT, and CRP levels were remarkably elevated in children with KD prior to treatment, which is a high-level inflammatory response in the acute phase of KD.

Corticosteroids are a relatively safe and affordable option for most individuals as adjunctive therapy for the primary treatment of KD. In the study by Burns[21], the author demonstrated an advantage in aneurysm size reduction among individuals treated with pulse methylprednisolone. This finding ensued global interest regarding steroid function in the treatment of acute KD.[21] A recent study showed that compared to IVIG alone, glucocorticoid combined with IVIG rapidly reduces TNF-α, IL-6, and CRP levels in individuals with KD.[13] Another prospective study showed that the addition of prednisolone to IVIG treatment reduced the inflammatory response in patients with KD.[15] In addition, this treatment regimen resulted in faster fever resolution than IVIG alone. This study confirmed similar results. With oral administration of aspirin, individuals exposed to glucocorticoids plus IVIG showed remarkably lower levels of inflammatory factors (such as TNF-α, IL-6, PCT, and CRP), compared to those who received conventional immunoglobulin therapy ($p < 0.05$). The rate of cumulative persistence of fever was also lower in the glucocorticoids plus IVIG category than in IVIG alone (log-rank, $p=0.024$). These findings strongly depict that the treatment regimen coupled with corticosteroids is more conducive to alleviating the inflammatory state and improving clinical symptoms in individuals with KD.

Prospective research revealed that anomalies in the coronary artery were significantly reduced in the IVIG plus prednisolone category than with IVIG alone. The authors suggest that the duration of steroid treatment for KD might be more relevant than the maximum concentration.[22] However, a subsequent study was unable to show a positive effect of steroid treatment on reducing CAA.[23] The reasons for the considerable variation between the two studies are (1) individuals with incomplete KD and (2) individuals with z-scores ≥2.5 for the initial coronary artery. In this study, the z-scores of coronary artery size post-treatment with IVIG + glucocorticoids were smaller relative to IVIG alone. However, these variations were not significant, probably due to the small sample size. Thus, further studies are needed to verify this finding. Although the difference was not

statistically significant, it indicates ways to mitigate CALs.

The PAC-1 monoclonal antibody identifies a conformational alteration in the GPIIb/IIIa complex on activated platelets.[24] This conformational change is a key step in platelet activation and leads to platelet aggregation by various pathways.[25] The vascular endothelial growth factor (VEGF) is released from platelets during whole blood clotting. It serves a crucial function in regulating angiogenesis.[19] Studies show that VEGF is highly expressed in the serum of children with KD during an early stage of vascular inflammation and is involved in the formation of CALs.[19,26] Platelet activation causes a conformational alteration in the GpIIb/IIIa complex. This, in turn, exposes almost 80,000 fibrinogen binding sites on the surface of the platelet.[27] Fibrinogen binding to these receptors might be a prerequisite for VEGF release.[28] Moreover, the activation of TLR4 leads to exacerbated platelet responses in KD patients, potentially contributing to atherogenesis through the delivery of proinflammatory factors to leukocytes and endothelial cells.[29,30] Furthermore, TLR4 induces nuclear factor-κB (NF-κB)-dependent cytokine production through the myeloid differentiation primary-response gene 88 (MyD88) pathway.[31,32] TLR4 signaling through NF-κB also contributes to the pathology of vascular injury in individuals with KD.[33]

Moreover, recent studies have shown that platelets are highly activated in KD patients, which is probably one of the most crucial pathophysiological steps in the disease.[1,34] Therefore, it is vital to assess whether there is any change in the status of platelets in KD patients post-treatment with glucocorticoid. Ueno and his colleagues[35] observed platelet activation levels as significantly elevated in individuals with KD and CAA versus individuals without CAAs. Additionally, platelet activation levels were considerably lower in individuals with KD exposed to both IVIG and oral prednisolone in comparison with individuals who had received IVIG alone.[35] The present study confirmed the presence of platelet activation in individuals with KD. It also found remarkably lower levels of PAC-1 and TLR4 in the glucocorticoid plus IVIG group relative to the control group ($p < 0.05$). These findings establish that reducing platelet activation is a function of glucocorticoids in KD. Furthermore, Yahata et al.[36] found that high levels of platelet activation were still present in children recovering from KD for 2-3 months, which may explain the better effect of long-term steroids than short-term steroids in children with KD.

Limited research has been conducted on the impact of glucocorticoids in reducing platelet activation. However, it is speculated that glucocorticoids function by inhibiting the pathway that stimulates platelet activation through the inflammatory response.[37] Glucocorticoid downregulates VEGF expression in the serum by inhibiting platelet activation, thereby suppressing vascular injuries in KD. Additionally, glucocorticoids also block the TLR4 signaling pathway by inhibiting platelet activation, which in turn, inhibits phosphorylation of NF-κB, thereby suppressing its activation. Furthermore, glucocorticoids interfere with the inflammatory response in KD by inhibiting inflammatory molecule production through the downregulation of NF-κB levels.[38]

Although this study demonstrates the use of glucocorticoids in KD treatment, the sample size was relatively small. Hence, there was no strict long-term follow-up of results, which limits the obtained insights. Future studies will need to expand the sample size and increase the post-treatment follow-up duration to accurately ascertain the long-term impact of glucocorticoids on the prognosis of individuals with heart damage.

In summary, glucocorticoids plus IVIG therapy may inhibit the inflammatory response and platelet activation and aid in vascular remodeling. Additional prospective studies

are needed to further investigate the efficacy of glucocorticoids plus IVIG therapy in the acute phase of KD.

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Ethical approval

The study was licensed by the Institutional Ethics Committee of the Provincial Children's Hospital and the Anhui Medical University. Additionally, signed, informed consent was obtained from all involved (Approval no: EYLL-2019-023).

Author contribution

The authors confirm their contribution to the paper as follows: study conception and design: QQW, LYZ, SZ; data collection: QQW; analysis and interpretation of results: QQW, SZ; draft manuscript preparation: QQW. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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