Vol 14, No 1 (2022)

Table of Contents

Review Article

Research Article

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RESEARCH ARTICLE

The Effect of Thymoquinone Administration on Local Immunoglobulin-G Levels of *Rattus norvegicus* Strain Wistar Sciatic Nerve Crush Injury Model

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Abstract

BACKGROUND: Immunoglobulin-G (IgG) is a product of the initial response to secondary immune response, which accumulates in distal segment of the nerve after crush injury. Thymoquinone modulates the adaptive immune response. Effect of thymoquinone administration on local IgG levels of *Rattus norvegicus* Wistar rats sciatic nerve crush injury model has not been elucidated.

METHODS: This was an experimental study, with 63 *Rattus norvegicus* Wistar rats that divided into 9 groups. Three groups were given placebo, 3 groups were given 100 mg/kg/day thymoquinone, and 3 groups were given 250 mg/kg/day thymoquinone. The rats were terminated based on the assigned group at 5x24, 6x24, and 7x24 hours and then the IgG levels were measured using sandwich enzyme-linked immunosorbent assay (ELISA).

RESULTS: There was a significant difference in IgG levels after administration of 100 and 250 mg/kg/day thymoquinone at 5x24 hours and 7x24 hours post-injury compared to the rats that were given no treatment. A significant difference of IgG levels was also found after administration of 100 mg/kg/day thymoquinone group at 6x24 hours post-injury. Critical point of decreasing local IgG of all groups happened at 6x24 hours after injury, however, there was no significant difference in the median levels of thymoquinone at doses of 100 mg/kg and 250 mg/kg.

CONCLUSION: Local IgG levels in distal segment of the sciatic nerve crush injury is lower in rats that were given 100 mg/kg thymoquinone treatment compared to the rats that receive no thymoquinone treatment since 5x24 hours after injury. Thymoquione administration should be given immediately after the crush injury until before 6x24 hours post-injury to decrease antibodies in degeneration process.

KEYWORDS: thymoquinone, immunoglobulin-G, crush injury, sciatic nerve

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Introduction

Crush injury is a peripheral nerve injury originating from acute traumatic compression due to a blunt object.(1) The sciatic nerve is one of the peripheral nerves of the lower extremity that is often injured.(2) The prevalence of sciatic nerve injury due to post-traumatic is 30%, due to fracture is 7.9-75%, and due to perioperative or postoperative is 5-15%.(2,3) Lesions to the axon and myelin sheath are seen in crush injury. Wallerian degeneration occurs in the distal segment from the lesion site of the injured peripheral nerve

axon.(4,5) This clearance of degenerated nerve myelin go through 2 phases. The first phase is mediated by Schwann cells, and the second phase is mediated by hematogenous phagocytes. Phagocytosis by macrophages is caused by opsonization of myelin debris by antibodies.(6)

Immunoglobulin-G (IgG) antibodies were observed to accumulate in the distal segment of the injured nerve on 6th day after crush injury. This indicates the presence of auto-antibodies in degenerated nerves, which help in cleaning up the myelin that degenerates rapidly.(6) The presence of this local IgG has a negative correlation with nerve regeneration.(7) IgG is a product of the initial response to secondary immune response and memory and therefore has important immune effects.(8)

One bioactive compound that able to modulate adaptive immune response is thymoquinone.(9) Thymoquinone is isolated from *Nigella sativa*, which is also known as black cumin.(10,11) Thymoquinone has beneficial antioxidant and anti-inflammatory effects, and also has shown to have neuroprotective effects by acting as a free radical scavenger for radicals released after traumatic nerve injury.(12) Oral administration of thymoquinone in rats at a dose of 10 to 100 mg/kg does not cause toxic or lethal effects. The maximum tolerated dose for oral administration of both male and female rats is 250 mg/kg.(13) The effect of immunity and dose in the administration of thymoquinone on local IgG levels of *Rattus norvegicus* strain Wistar sciatic nerve crush injury model has not been elucidated before. Therefore, in this study we tried to determine the effect of thymoquinone in local deposition of IgG in injured peripheral nerves.

Methods

Study Design and Animal Model

This was an experimental study, with 63 *Rattus norvegicus* male Wistar rats aged 18-20 weeks old age and weighted 300-350 grams. Thirty days prior to the treatment, each rat was given food and drink ad libitum, and kept in the cage for 12 hours in light and 12 hours in dark each day. The room temperature was set between 22-25°C. Measurements of body weight and Sciatic Functional Index (SFI) values of all rats were carried out a day before the crush injury procedure to assure that all the rats were in accordance with the desired criteria.

Administration of anesthetic ketamine and xylazine via intraperitoneal injection were given to the rats before the crush injury procedure.(14) Anatomical pathological examination was carried out before the selection of forceps, clamping position, and degree of clamping of the hook to ensure that the degree of nerve lesion was the intended crush injury. The sciatic nerve, which was located 10 mm superior of left sciatic nerve trifurcation, was carefully separated from the surrounding tissue and then compressed by Kelly hemostatic forceps for 30 seconds (Figure 1).(15,16) This study was declared ethically feasible by the Health Research Ethics Committee, Faculty of Medicine, Airlangga University, Surabaya (No. 4/EC/KEPK/FKUA/2021).

Experimental Intervention

Sixty-three rats were divided into 9 groups, with each group consist of 7 rats. Three groups were given corn oil as a placebo (Group A1, A2, and A3), 3 groups were given thymoquinone 100 mg/kg/day (Group B1, B2, and B3), and 3 groups were given thymoquinone 250 mg/kg/day (Group C1, C2, and C3). The thymoquinone was obtained from Santa Cruz Biotechnology, Inc (Dallas, TX, USA), with the purity level of the material ≥98% and was dissolved in corn oil.

The treatments were given at different doses according to the group as soon as the rats woke up from anesthesia after crush injury procedure, orally via an orogastric tube. Later, the treatments were routinely given once a day until the rats were euthanized at the end of the follow-up period. Groups A1, B1, and C1 were terminated at 5x24 hours after crush injury. Groups A2, B2, and C2 were terminated at 6x24 hours after crush injury. Groups A3, B3, and C3 were terminated at 7x24 hours after crush injury.

IgG Measurement

The IgG levels were measured at 5 mm from the distal nerve lesion. The sciatic nerve tissue was homogenized in 600 µL PRO-PREP solution. Furthermore, cell lysis was induced by incubation for 20-30 minutes in a refrigerator at -20°C. Centrifugation was carried out at 13,000 rpm at 4°C for 5

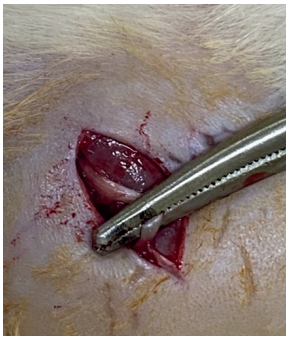

Figure 1. The compression of the sciatic nerve at a position 5 mm from the tip of the forceps.

minutes. The supernatant of the sample was then transferred to a 1.5 mL tube.

IgG levels were measured using the sandwich enzyme-linked immunosorbent assay (ELISA) method with HumaReader Single (Human Diagnostic, Wiesbaden, Germany). Meanwhile the reagent used was rat IgG ELISA Kit (No Cat. E-EL-R0518) from Elabscience (Wuhan, China). As much as 100 µL samples were added to the micro-ELISA plate wells which had been pre-coated with an antibody specific to Rat IgG. Then a biotinylated detection antibody specific for Rat IgG and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. After the substrate solution was added to each well, the wells that contain Rat IgG, biotinylated detection antibody and Avidin-HRP conjugate would appeared blue in color. The optical density (OD) was measured spectrophotometrically at a wavelength of 450±2 nm. The OD value was proportional to the concentration of Rat IgG, with the sensitivity of 1.88 ng/mL.(17)

Statistical Analysis

Statistical analysis was performed using SPSS ver 21.0 (IBM Corporation, Armonk, NY, USA). Levene's test was conducted to determine the homogeneity of body weight SFI values. The Shapiro-Wilks test was conducted to determine the normality of the data in each treatment group and time group. Furthermore, a comparison test between treatment groups using Kruskal-Wallis and Mann-Whitney test was carried out. The Wilcoxon and Friedman signed ranks test were also performed to determine the critical point of decreasing local IgG of all groups.

Results

Homogeneity and Normality Test Results

For the calculation of the homogeneity and normality test, the groups were distributed into 5x24 Hours group (A1, B1, and C1), 6x24 Hours group (A2, B2, and C2), and 7x24 Hours group (A3, B3, and C3). The results showed that *p*-value for rats' body weight was 0.544 and the SFI value was 0.919 (Table 1). These results indicated that body weight and SFI values were homogeneous.

Meanwhile for the normality test, *p*-value for the 5x24 Hours group, 6x24 Hours group, and 7x24 Hours group were 0.000, 0.036, and 0.781, respectively (Table 2). Beside the grouping based on the rats' termination period, for the normality test the data were also classified into different treatment group type, namely Control group (A1, A2, and A3), 100 mg Thymoquinone group (B1, B2, and B3), and 250 mg Thymoquinone group (C1, C2, and C3). The *p*-value for Control group, 100 mg Thymoquinone group, and 100 mg Thymoquinone group were 0.001, 0.020, and 0.454, respectively (Table 3). The two results of the normality test of showed that the data distribution were not normal in all treatment groups. Based on the results of the normality test of the data in Table 2 and Table 3, non-parametric statistical tests were then carried out.

IgG Level

Analysis of IgG levels was determined based on the median value of IgG levels in each treatment group. The median value of IgG levels in the Control group was higher than both Thymoquinone group throughout the study. The biggest difference occurred in 5x24 hours after the crush injury performed, which showed the median IgG levels in Control, 100 mg Thymoquinone, and 250 mg Thymoquinone groups were 17.47 ng/mL, 9.57 ng/mL, and 9.30 ng/mL, respectively.

The median value of IgG levels in the 100 mg Thymoquinone group was lower than the 250 mg Thymoquinone group at 6x24 and 7x24 hours after crush injury. IgG levels in 100 mg Thymoquinone and 250 mg Thymoquinone groups at 6x24 hours after crush injury were 5.57 ng/mL and 5.95 ng/mL, respectively. Meanwhile, the IgG levels in 100 mg Thymoquinone and 250 mg

Table 1. Homogeneity test for body weight and SFI values of subjects.

	Group	Range	Mean±SD	*p*-value
Body Weight (gram)	5x24 Hours	302 – 348	324.62± 13.84	0.544
	6x24 Hours	302 – 348	326.90± 12.34	
	7x24 Hours	302 – 346	325.29± 13.55	
SFI (cm)	5x24 Hours	(-7.06) – (-0.98)	-4.02± 1.73	0.919
	6x24 Hours	(-6.98) – (-0.52)	-3.71± 1.68	
	7x24 Hours	(-6.04) – (-0.52)	-3.53± 1.59	

*The homogeneity was determined by Levene's.

Table 2. Normality test for each time group.

Variable	Group	Statistics	*p*-value
IgG	5 x 24 Hours	0.788	0.000
	6 x 24 Hours	0.901	0.036
	7 x 24 Hours	0.977	0.781

*The homogeneity was determined by Shapiro-Wilks test.

Table 3. Normality test for each treatment group.

Variable	Treatment	Statistics	*p*-value
IgG	Control	0.814	0.001
	100 mg Thymoquinone	0.887	0.02
	250 mg Thymoquinone	0.957	0.454

*The homogeneity was determined by Shapiro-Wilks test. Control: Crush injury (+), corn oil (+), thymoquinone (-); 100 mg thymoquinone: crush injury (+), corn oil (+), thymoquinone 100 (+); 250 mg thymoquinone crush injury (+); corn oil (+); thymoquinone 250 (+).

Thymoquinone at 7x24 hours after crush injury were 6.38 ng/mL and 7.31 ng/mL, respectively (Figure 2).

The differences in IgG levels were analyzed using the Kruskal-Wallis test then followed by the Mann-Whitney test. The results of the Kruskal-Wallis test showed *p*-value of 0.012, 0.038, and 0.008 for 5x24 Hours, 6x24 Hours, and 7x24 Hours group, respectively (Table 4), which mean that there were different effect on the treatment of no thymoquinone, 100 mg/kg thymoquinone, and 250 mg/kg thymoquinone in rats that were terminated at 5x24 hours, 6x24 hours, and 7x24 hours after the crush injury performed. Since the result of Kruskal-Wallis showed significance for all groups ($p<0.05$), so a follow up test was carried out. The Mann-Whitney test showed that there was a significant difference of IgG levels in 100 mg Thymoquinone and 250 Thymoquinone groups at 5x24 hours ($p=0.007$, $p=0.011$, respectively) and 7x24 hours ($p=0.017$, $p=0.002$) post-injury compared to the Control group. Different results were obtained in the 6x24 hour post-injury period. During this period, a significant difference of IgG level was only found in the 100 mg thymoquinone group compared to the Control group ($p=0.026$). Meanwhile, there was no significant difference in 100 mg Thymoquinone and 250 mg Thymoquinone groups at 5x24 hours, 6x24 hours, and 7x24 hours post-injury (Table 4).

IgG Decrease Critical Point

The Wilcoxon signed ranks test showed that the critical point of decreasing local IgG of all groups happened at 6x24 hours after the crush injury performed ($p=0.018$). This was followed by a Friedman test, which showed that Control group ($p=0.001$), 100 mg Thymoquinone group ($p=0.006$) and 250 mg Thymoquinone group ($p=0.006$) were all significant.

Discussion

A literature report that IgG antibodies were observed to accumulate in the distal segment of the injured nerve on the 6th day after crush injury (6), hence our current study was based on this founding. We terminated the experimental rats at 5x24 hours and 7x24 hours as buffers to complement the 6x24 hours duration, to know whether the administration of thymoquinone would effectively be able to lower the accumulate IgG levels on rats after the crush injury.

The results of this study showed a significant difference in IgG levels in the 100 mg Thymoquinone and

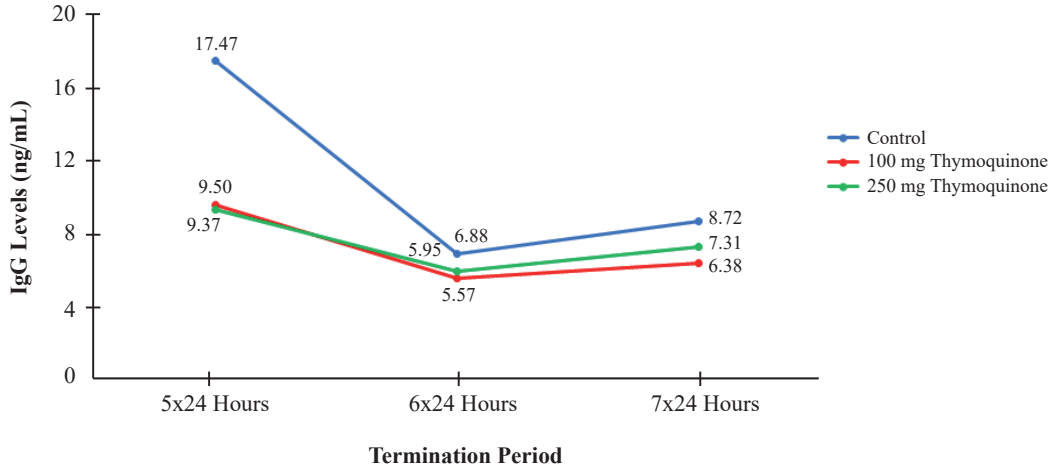

Figure 2. Descriptive analysis of IgG levels.

Table 4. Kruskal-Wallis and Mann-Whitney test for IgG levels (ng/mL) after crush injury.

Termination Period	Treatment	Median	*p*-value*	*p*-value#		
				1 and 2	1 and 3	2 and 3
5 x 24 Hours	1	17.47	0.012	0.007	0.011	1
	2	9.57				
	3	9.3				
6 x 24 Hours	1	6.88	0.038	0.026	0.053	0.318
	2	5.57				
	3	5.95				
7 x 24 Hours	1	8.72	0.008	0.017	0.002	0.259
	2	6.38				
	3	7.31				

*Tested with Kruskal-Wallis, significant if $p<0.005$; #Tested with Mann-Whitney, significant if $p<0.005$. 1: Control group; 2: 100 mg Thymoquinone; 3: 250 mg Thymoquinone.

250 mg Thymoquinone at the 5x24 hours and 7x24 hours post-injury compared to the Control group. A significant difference of IgG levels was also found in the 100 mg Thymoquinone group at 6x24 hours after the crush injury. The level of IgG levels in both the 100 mg Thymoquinone and 250 mg Thymoquinone throughout the study were also found to be lower than the Control group which receive no thymoquinone treatment. These show that local IgG levels in distal segment to peripheral nerve lesions decrease after the thymoquinone administration.

This is assumed due to the inhibitory effect of Th2 cytokines produced by thymoquinone.(18) Th2 cells produce interleukin (IL)-4, IL-5, IL-10, and IL-13 cytokines that play important roles in the induction of humoral immune responses. Not only activates macrophage M2, cytokines IL-4/IL-13 but also activates B cell proliferation, immunoglobulin class changes, and antibody production. (19,20) IL-4 stimulates the secretion of certain Ig, such as IgG1 and IgE (21), meanwhile IL-10 is known to be able to activate B cell to produce IgG and IgM (22). Local IgG deposits are intended for the clearance of rapidly degenerating myelin.(6) Nonetheless, immunoglobulin synthesis and accumulation as well as immune cell infiltration are said to have an inhibitory effect on neuronal regeneration.(8)

The Friedman and the Wilcoxon signed ranks test of this study showed that the critical point of decreasing local IgG after the thymoquinone administration of all groups happened at 6x24 hours after the crush injury ($p=0.018$). This result supported hypothesis that the administration of thymoquione must be given immediately after the crush injury until before 6x24 hours post-injury, prior to the accumulation of IgG levels. However, there was no significant difference in the median levels of thymoquinone at doses of 100 mg/kg and 250 mg/kg. From these results it can be assumed that the low level of local IgG levels was not affected by the addition of thymoquinone dose. Hence, the administration of thymoquinone at a dose of 100 mg/kg has enough potential to lower IgG levels compare to the rats that receive no thymoquinone treatment. These results are consistent with another study which concluded that increasing the dose of thymoquinone did not change the effect.(23)

Antibodies are required for wound healing and debris elimination in peripheral nerve crush injury. On the other hand, the autoantigenic antibodies of the autoimmune group most often originate from the IgG isotype.(24) This depends on the IgG subclass (isotype) and the glycosylation/sialylation pattern. This property modulates antibody binding to different types of Fc-receptors from natural effector cells. Auto-antibody glycosylation differences are important regulators of autoimmune disorders.(25) Research to determine the mechanism pathway of the effect of thymoquinone on Th2 cells, IL-4 cytokines, and subclass variables (isotypes) as well as IgG glycosylation/sialylation patterns in the distal segment of sciatic nerve crush injury lesions is necessary to be further developed.

The limitation of this study was the short observation time, since we only observe until the 7th day. A study reported that although IgG was found in the distal segment of the injured nerve on the 6th day after crush injury, serum IgG levels increased after 21 days of onset. The delay may be because the generation of IgG in the blood requires antigen uptake, processing, and presenting; since the production of IgG antibodies is influenced by cytokines secreted by Th2 cells. At the onset of peripheral nerve injury, there is a temporary decrease in immune status. T cells will return to normal level after 14 days of peripheral nerve injury.(8)

Therefore, it will be better if additional observation of 14 days was conducted to evaluate the effect of thymoquinone administration on local IgG levels in the distal segment of the lesion.

Conclusion

The local IgG antibody levels in the distal segment of the sciatic nerve crush injury is lower in rats that were given thymoquinone treatment with the dose of 100 mg/kg/day compared to the rats that receive no thymoquinone treatment since 5x24 hours after injury. Hence the administration of thymoquione should be given immediately after the crush injury until before 6x24 hours post-injury to decrease antibodies in degeneration process.

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Authors Contribution

VB, AHB, and MHM were involved in planning and supervising the research. VB, JN, and PBN were involved in sample processing and measurement of IgG levels. VB, PBN, and BU were involved in data processing, statistical analysis and calculations, and compiling tables and figures. VB, AHB, JN, and BU were involved in the preparation of the manuscript. VB, AHB, JN, BU, and PBN were involved in the discussion of the results and comments on the manuscript.

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