TurnitinForkheadBoxP3MessengerRNA

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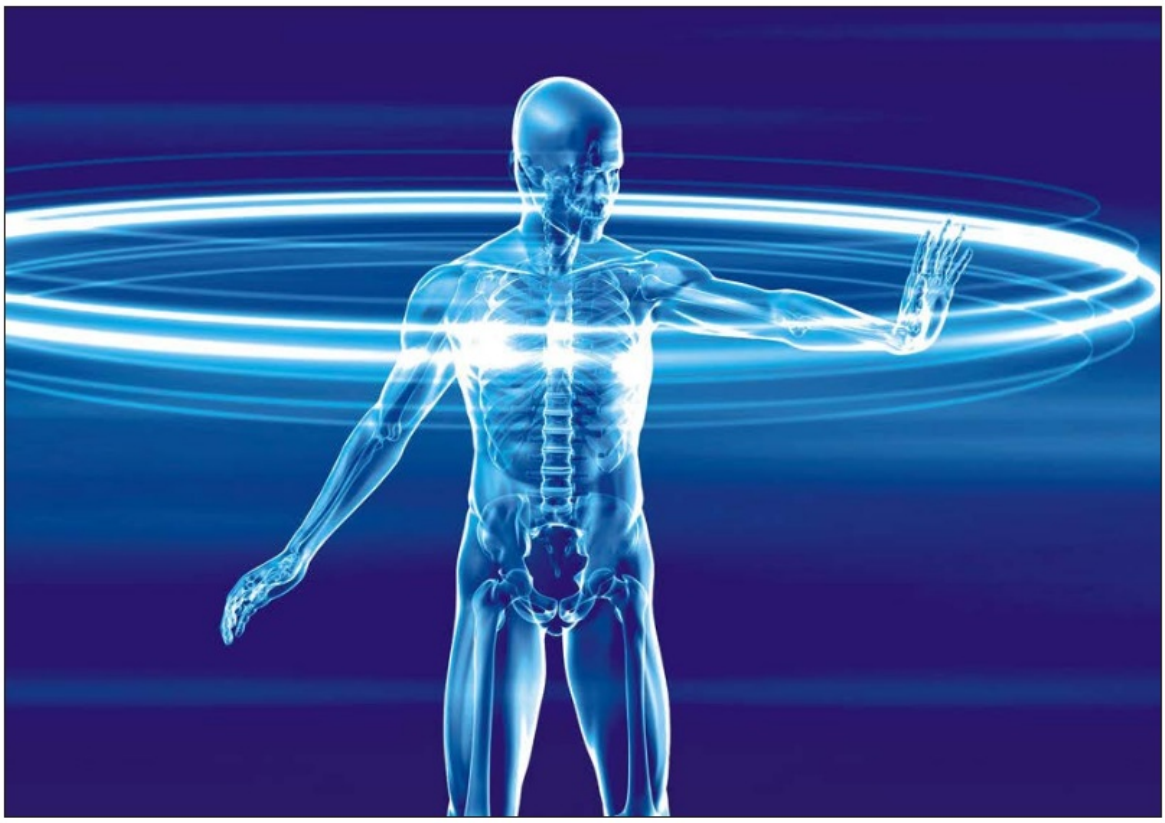

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Research Article
Forkhead Box P3 Messenger-RNA Expression after *Curcuma longa* Extract Intervention in Early Pregnant Mice with Toxoplasmosis

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Abstract

Background and Objective: *Curcuma longa* (*C. Longa*) has strong anti-inflammatory effect. This study aims to examine the effect of *Curcuma longa* extract on Forkhead Box P3 (FOXP3) mRNA expression in early pregnant mice with acute toxoplasmosis. **Materials and Methods:** This study evaluated 20 early pregnant mice that were divided into 5 groups, four mice in each. Group 1-4 received injections of *Toxoplasma gondii* tachyzoites. Three days later, G1 and G2 were given orally 125 and 500 mg kg^{-1}/day of *Curcuma longa* extract, respectively. The G3 was given 60 mg kg^{-1}/day of spiramycin (positive control) and G4 was given 0.2 mL of distilled water (negative control). The G5 underwent no intervention at all. Blood samples were obtained serially (before and 3 days after injection of tachyzoites, 3 and 7 days after intervention) to assess FOXP3 mRNA expression. **Results:** The FOXP3 mRNA expression increased significantly in G1-G3 3 days after intervention ($p<0.05$), whereas, FOXP3 mRNA expression decreased significantly ($p<0.05$) 7 days after intervention and there was no significant difference between these three groups. The FOXP3 mRNA expression in G4 increased significantly 3, 6 and 10 days after tachyzoites injections, while FOXP3 mRNA expression on G5 fluctuated but considered as insignificant ($p>0.05$). **Conclusion:** The administration of *Curcuma longa* extract at a dose of 125 mg kg^{-1}/day for 3 days effectively increased FOXP3 mRNA expression and 7 days administration resulted in decreased FOXP3 mRNA expression in early pregnant mice with acute toxoplasmosis.

Key words: FOXP3 mRNA, *Curcuma longa*, pregnancy, *Toxoplasma gondii*, spiramycin

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Toxoplasmosis is a zoonotic disease caused by the parasite *Toxoplasma gondii*[1]. Prevalence of toxoplasmosis is different among country. Gandahusada[2] reported that the incidence of toxoplasmosis in Indonesia is 2-63%. The latest incidence in Indonesia, particularly in Jakarta and Surabaya was reported as 70 and 58%, respectively[3]. According to Malarvizhi[4], the incidence of toxoplasmosis in pregnancy in Asia and Europe was: Korea (0.8%), Vietnam (11.2%), Sudan (34.2%), New Zealand (33%), Kuba (70.9%) and Europe (9-67%).

Humans can be infected through oral or fecal transmission, blood transfusion containing tachyzoites and vertical infections from mother to fetus through placenta[5]. Toxoplasmosis becomes important in obstetrics because acute *Toxoplasma gondii* (*T. gondii*) infection during early pregnancy can lead to congenital abnormalities, fetal death and abortion[6].

The Forkhead box p3 (FOXP3) gene is the main regulator of $CD4^+CD25^+$T cells (Treg cells)[7]. The *FOXP3* gene plays an important role in maintaining the body's immune balance, thereby preventing cell damage due to excessive inflammatory reactions[8]. Increased FOXP3 mRNA expression may cause various diseases[9]. The FOXP3 mRNA expression decreases in the presence of acute toxoplasmosis. Thus such decreased in FOXP3 mRNA expression causes abortion in early pregnant mice with acute toxoplasmosis[10].

Curcuma longa is rhizomatous herbaceous perennial plant of the Ginger family. Curcumin is the major component of C. *longa*[11]. Several studies have been done to prove the effectiveness of Curcumin. *Curcuma longa* extract has been shown to be an anti-inflammatory agent[12]. Curcumin was also prove to be anti-bacterial[13], anti-paracite[14], anti-cancer[15] and against body's immune system-related-disease[15].

Curcuma longa is one of the home spices that were found and used extensively in Asia especially in Indonesia. *Curcuma longa* were also studied and proved to be potent anti-inflammatory, anti-microbial and anti-oxidant. Based on this rationale, authors aim to initiate research on effectiveness of the *C. longa* extract affecting FOXP3 mRNA expression in early pregnant mice with acute infection of *T. gondii* (acute toxoplasmosis), hoping that the result will provide additional information for further research.

MATERIALS AND METHODS

This study was conducted in Molecular Biology and Immunology Laboratory, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia for 5 months (June, 2017 until October, 2017). Pathogen free female Balb/c mice were obtained from Animal Laboratory Bogor, West Java, Indonesia. Experimental animals were chosen according to research guidelines for evaluating the safety and efficacy of herbal medicines under WHO standard. Mice were handled in Animal Laboratory, Faculty of Medicine, Hasanuddin University and Makassar, Indonesia in specified room with adequate air flow under standard room temperature (28±2°C). Mice were fed with standard natural pellets and were given adequate amount of water.

Toxoplasma gondii tachyzoites strain RH suspension were obtained from the Laboratory of Bogor Agriculture Institution, West Java, Indonesia and were stored in freezer under -20°C before usage.

This study was conducted using 20 healthy female Balb/c mice (age 11-13 weeks, weight 16-20 g, active and have a good appetite). The early pregnant mice were divided into five groups (G1-G5) randomly, 4 mice each group. The G1-G4 were injected with 10 tachyzoites of *T. gondii* RH strain intraperitoneal and G5 without infection. Three days after injection of tachyzoites, G1 and G2 were each given *C. longa* extract 125 and 500 mg kg^{-1}/day, respectively, G3 (positive control) was given spiramycin 60 mg kg^{-1}/day and G4 (negative control) was given 0.2 mL of distilled water. Each intervention was administered orally using cannulas for 7 days. G5 underwent no intervention at all.

Blood samples were taken from the tail vein serially (1 day before tachyzoites injection, 3 days after tachyzoites injection, 3 and 7 days after the intervention). The FOXP3 mRNA expression was evaluated using the quantitative Polymerase Chain Reaction (qPCR) technique. Quantitative PCR systems (Applied Biosystems, Foster City, CA, USA) used Power SYBR® Green PCR Mix (Applied Biosystems). The primary FOXP3 used was FW-TTTACTCGCATGTTGCCTACTT and RV-TCAAATTCATCTACGGTCCACACT (NM_001199347.1), with standards of GAPDH FW-CATGGCCTTCCGTGTTCCT and RV-GCGGACGTCAGATCCA (M32599.1) normality. Anti-toxoplasma IgG/IgM antibody levels were determined by ELISA method, using a qualitative mouse antibody IgG (TP-IgG) ELISA kit (Cat No: MBS 109093) and a qualitative mouse antibody IgM (TP-IgM) ELISA kit (Cat No: MBS 9310461).

At the end of the study, the mice were sacrificed then buried in specified places. The procedures for animal preservation and intervention in this study follow the procedures of the UK Animal (Scientific Procedures) Act, 1986 and associated guidelines[16]. This study has been approved by the animal ethics research committee of the Faculty of Medicine, University of Hasanuddin, Makassar, Indonesia.

Research data were obtained, tabulated and processed by computer using SPSS version 20 software. The data were analyzed statistically using paired t-test and one-way ANOVA test with significance level of p<0.05.

RESULTS

The FOXP3 gene expression before and after tachyzoites injection are shown in Table 1. The FOXP3 gene expression 3 days after injection decreased significantly (p<0.05) compared to uninfected group as shown in Table 2. The FOXP3 gene expression increased significantly (p<0.05) 3 days after intervention with *C. longa* extract at dose of 125 mg kg^{-1}/day (G1), C. *longa* at dose of 500 mg kg^{-1}/day (G2) and spiramycin at dose of 60 mg kg^{-1}/day (G3). The increased in FOXP3 gene expression among these 3 groups (G1, G2, G3) does not differ significantly as shown in Table 3. FOXP3 gene expression decreased significantly (p<0.05) 7 days after intervention with *C. longa* extract at dose of 125 mg kg^{-1}/day (G1), *C. longa* at dose of 500 mg kg^{-1}/day

Table 1: FOXP3 mRNA expression before and after injection of tachyzoites and after intervention

	FOXP3 mRNA expression			
Groups	A	B	C	D
G1	9.98±0.14	7.98±0.21	12.03±0.13	9.88±0.23
G2	9.96±0.14	8.07±0.17	12.12±0.32	9.87±0.13
G3	10.02±0.15	8.54±0.09	11.93±0.12	10.14±0.15
G4	10.18±0.12	8.85±0.15	7.09 ±0.15	6.12±0.14
G5	10.00±0.19	10.20±0.14	10.06 ±0.19	9.90 ±0.16

±(Standard deviation value). Early pregnant mice group (G), G1-G4 were given *Toxoplasma gondii* tachyzoites injection. While G5 were not injected and G1 was given *Curcuma longa* extract 125 mg kg^{-1}/day, G2 was given *Curcuma longa* extract 500 mg kg^{-1}/day, G3 was given spiramycin 60 mg kg^{-1}/day, G4 was given distilled water 0.2 mL/day, G5 underwent no intervention. Time of sample collection, A: 1 day prior tachyzoites injection, B: 3 days after tachyzoites injection, C: 3 days after the intervention and D: 7 days after the intervention

Table 2: FOXP3 gene expression dynamics before and after *Curcuma longa* extract intervention and control groups

	Paired differences							
				95% confidence interval of different				
Group (G)/ pair group	Mean	Std. Dev	Std. error mean	Lower	Upper	t	df	Sig. (2-tailed)
G1								
FOXA-FOXB	1.945	0.20936	0.10468	1.61185	2.27815	18.580	3	0.000
FOXB-FOXC	-4.000	0.13491	0.06745	-4.21467	-3.78533	-59.300	3	0.000
FOXB-FOXD	-1.845	0.29103	0.14552	-2.30810	-1.38190	-12.679	3	0.001
FOXC-FOXD	2.155	0.36060	0.18030	1.58120	2.72880	11.952	3	0.001
G2								
FOXA-FOXB	1.8725	0.08421	0.04211	1.73850	2.00650	44.471	3	0.000
FOXB-FOXC	-4.0575	0.44124	0.22062	-4.75961	-3.35539	-18.391	3	0.000
FOXB-FOXD	-1.8025	0.68427	0.34213	-2.89133	-0.71367	-5.268	3	0.013
FOXC-FOXD	2.2550	0.27815	0.13907	1.81240	2.69760	16.214	3	0.001
G3								
FOXA-FOXB	1.4325	0.38239	0.19120	0.82403	2.04097	7.492	3	0.005
FOXB-FOXC	-3.3625	0.39016	0.19508	-3.98333	-2.74167	-17.237	3	0.000
FOXB-FOXD	-1.5500	0.49686	0.24843	-2.34061	-0.75939	-6.239	3	0.008
FOXC-FOXD	1.8125	0.25461	0.12730	1.40736	2.21764	14.238	3	0.001
G4								
FOXA-FOXB	1.8350	0.11504	0.05752	1.65195	2.01805	31.903	3	0.000
FOXB-FOXC	1.2625	0.17652	0.08826	0.98162	1.54338	14.305	3	0.001
FOXB-FOXD	2.2275	0.40681	0.2034	1.58018	2.87482	10.951	3	0.002
FOXC-FOXD	0.9650	0.25146	0.12573	0.56487	1.36513	7.675	3	0.005
G5								
FOXA-FOXB	-0.0125	0.37349	0.18674	-0.60680	0.58180	-0.067	3	0.951
FOXB-FOXC	0.0175	0.15108	0.07554	-0.22290	0.25790	0.232	3	0.832
FOXB-FOXD	0.2925	0.34856	0.17428	-0.26213	0.84713	1.678	3	0.192
FOXC-FOXD	0.2750	0.41741	0.20871	-0.38920	0.93920	1.318	3	0.279

Early pregnant mice group (G), G1-G4 were given *Toxoplasma gondii* tachyzoites injection. While G5 were not injected and G1 was given *Curcuma longa* extract 125 mg kg^{-1}/day, G2 was given *Curcuma longa* extract 500 mg kg^{-1}/day, G3 was given spiramycin 60 mg kg^{-1}/day, G4 was given distilled water 0.2 mL/day, G5 underwent no intervention. FOXA: FOXP3 gene expression 1 day before tachyzoites injection, FOXB: FOXP3 gene expression 3 days after tachyzoites injection, FOXC: FOXP3 gene expression 3 days after intervention, FOXD: FOXP3 gene expression 7 days after intervention, Significant value: p<0.05, Std. Dev: Standard deviation

Table 3: Paired group comparison of FOXP3 gene expression

Dependent variables	(I) Group	(J) Group	Mean difference (I-J)	Std. Error	Sig.	95% confidence interval	
						Lower bound	Upper bound
FOXP3 gene expression 3 days after *C. longa* intervention	*C. longa* 125 mg kg^{-1}/day	*C. longa* 500 mg kg^{-1}/day	-0.08750	0.16967	0.984	-0.6114	0.4364
		Spiramycin 60 mg kg^{-1}/day	0.08250	0.16967	0.987	-0.4414	0.6064
		Placebo	4.94750*	0.16967	0.000	4.4236	5.4714
		Normal	1.86000*	0.16967	0.000	1.3361	2.3839
	C. longa 500 mg kg^{-1}/day	*C. longa* 125 mg kg^{-1}/day	0.08750	0.16967	0.984	-0.4364	0.6114
		Spiramycin 60 mg kg^{-1}/day	0.17000	0.16967	0.850	-0.3539	0.6939
		Placebo	5.03500*	0.16967	0.000	4.5111	5.5589
		Normal	1.94750*	0.16967	0.000	1.4236	2.4714
FOXP3 gene expression after 7 days *C. longa* intervention	*C. longa* 125 mg kg^{-1}/day	*C. longa* 500 mg kg^{-1}/day	0.01250	0.26914	1.000	-0.8186	0.8436
		Spiramycin 60 mg kg^{-1}/day	-0.26000	0.26914	0.866	-1.0911	0.5711
		Placebo	3.75750*	0.26914	0.000	2.9264	4.5886
		Normal	-0.02000	0.26914	1.000	-0.8511	0.8111
	C. longa 500 mg kg^{-1}/day	*C. longa* 125 mg kg^{-1}/day	-0.01250	0.26914	1.000	-0.8436	0.8186
		Spiramycin 60 mg kg^{-1}/day	-0.27250	0.26914	0.846	-1.1036	0.5586
		Placebo	3.74500*	0.26914	0.000	2.9139	4.5761
		Normal	-0.03250	0.26914	1.000	-0.8636	0.7986

Significant value: p<0.05, Std. Error: Standard error

(G2) and spiramycin at dose of 60 mg kg^{-1}/day (G3). The decreased in FOXP3 gene expression among these 3 groups (G1, G2, G3) does not differ significantly (Table 3). The FOXP3 gene expression in G5 fluctuated insignificantly during the experiment.

DISCUSSION

The anti-toxoplasma IgG/IgM antibody levels were measured before and 3 days after injection of the *T. gondii* tachyzoites. Anti-toxoplasma IgG/IgM antibody levels increased significantly 3 days after the tachyzoites injection (data not shown). In the previous study, intraperitoneal injection of 10 T. *gondii* tachyzoites in early pregnant mice lead to remarkable increase of anti-toxoplasma IgM antibody level after 24 h, whereas, IgG antibody level increase significantly after 72 h[17].

In this study, FOXP3 mRNA expression decreased significantly ($p<0.05$) 3 days after tachyzoites injection, continue to decline until day 6 and day 10 in G5 (the pregnant group, which was not given the tachyzoites injection and no intervention). Some investigators also reported decreased FOXP3 mRNA expression on spleen and placenta of pregnant mice infected with *T. gondii*[7,18].

The *FOXP3* gene's regulates CD4$^+$CD25$^+$ T cells (Treg cells). Treg cells help to regulate the immunological balance[19]. Decreasing FOXP3 expression is closely related to abortion[20-22]. The declining FOXP3 mRNA expression during acute infection will decrease suppressive effects of CD4$^+$CD25$^+$T cells on T helper cells[6], especially Th1[22,23]. The reduction of suppressive effect of Th cells causes increased pro-inflammatory cytokines (e.g., interferon γ, TNF-α)[24]. Pro-inflammatory action increases when there is infection but an excessive increase in pro-inflammatory Th1 may cause intravascular thrombosis and cellular damage. When this happens in early pregnancy, abortion may occur[25,26].

Three days after the interventions with 125 and 500 mg kg^{-1}/day of *C. longa* extract, respectively and 60 mg kg^{-1}/day of spiramycin, the FOXP3 mRNA expression increased significantly ($p<0.05$), although there was no significant difference between these three groups ($p>0.05$). Cong *et al.*[27] reported that FOXP3 gene expression increased after induction with curcumin. They found that FOXP3 gene expression increased over time (6, 24 and 48 h and 7 days) following induction with curcumin.

Curcumin, a major component of *C. longa*[28,29], may stimulate the FOXP3 mRNA expression[12,28]. Zhao *et al.*[30] reported that curcumin has an inhibitory effect on CD4$^+$CD25$^+$ T-cell suppressor activity. It increases FOXP3 mRNA expression via the transformation growth factor β pathway. The activated T cells suppress T-helper cells, so the expression of pro-inflammatory cells, especially Th1, is not too high[27,31].

Seven days after the intervention with 125 and 500 mg kg^{-1}/day of *C. longa* extract and 60 mg kg^{-1}/day of spiramycin, the FOXP3 mRNA expression decreased significantly ($p<0.05$), although there was no significant difference among these three groups ($p>0.05$). Present results are different from those reported by Cong *et al.*[27], who stated that the expression of FOXP3 gene increased periodically up to 7 days after the curcumin intervention. Zhao *et al.*[12] reported that administering curcumin at 200 mg kg^{-1}/day for 7 days increased T-cell secretion.

The result of this study provides new information about FOXP3 gene expression dynamics on early pregnant mice with acute toxoplasmosis that intervened with *C. longa* extract. Current research is limited with small sample size so further research with larger sample size.

CONCLUSION

Curcuma longa extract intervention has stable influences on dynamics of FOXP3 mRNA expression in early pregnant mice with acute toxoplasmosis. The administration of *C. longa* extract at a dose of 125 mg kg^{-1}/day for 3 days increased FOXP3 mRNA expression significantly ($p<0.05$) and administration during 7 days decreased FOXP3 mRNA expression significantly ($p<0.05$).

SIGNIFICANCE STATEMENT

This study was carried out on early pregnant mice with acute toxoplasmosis with intention to prove *C. longa* extract on up-regulation of FOXP3 gene expression. The FOXP3 gene expression is known to decrease on acute toxoplasmosis. Such decreased in FOXP3 gene expression causes abortion in early pregnant mice with acute toxoplasmosis. This study will help the researchers to uncover the critical areas on the effect of *C. longa* extract to prevent abortion on early pregnancy with acute *Toxoplasma gondii* infection by interfering up-regulation pathway of FOXP3 gene that haven't explored by many researchers. Thus, the result of this study can provide latest information for further research.

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