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Green tea extract resulted in a lower value of IL-6 and TNF-α expressions on rabbit model with Anterior Cruciate Ligament (ACL) rupture

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ABSTRACT

Background: Anterior Cruciate Ligament (ACL) rupture often occur at a young age. There is still debate about the timing of the surgical interventions. Many studies recommended that ACL reconstruction should be done after the third week of injury. Provision of green tea extract on ACL rupture is expected to more rapidly reduce the inflammatory reaction, which is characterized by lower IL-6 levels and lower TNF-α expression at the first, second and third week as compared with no administration of green tea extract so that surgical intervention can be done earlier.

Aim: To evaluate the effect of green tea extract in IL-6 expression and TNF-α level.

Methods: This research is an experimental randomized post-test only group design with 24 ACL-transected rabbit samples and divided into six groups. Three treatment groups and three control groups were further divided into first, second, and third week groups. The treatment group was administered with 300mg/kgBW green tea extract and control group was not administered. IL-6 and TNF-α were examined in each treatment and control group during the first, second and third weeks.

Results: Oneway-ANOVA study showed that serum IL-6 in the treatment group was lower as compared to control group at first, second, and the third week with $p = 0.000$ ($p < 0.05$). Kruskal-Wallis study for TNF-α expression in the treatment group was lower than the control group at first, second, and the third week with $p = 0.000$ ($p < 0.05$).

Conclusion: Green tea extract resulted in lower IL-6 expression and lower TNF-α level than with those not administered green tea extract.

Keywords: ACL rupture, inflammation, green tea extract, IL-6, TNF- α

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BACKGROUND

Anterior Cruciate Ligament (ACL) rupture is one of the most common injuries occurring in young productive age. The need for faster recovery time affects ACL reconstruction surgical interventions. In post rupture of the ACL, the resulting pathological changes are mediated by pro-inflammatory mediators such as IL-1β, IL6, TNF-α. These mediators will cause damage to the cartilage and inflammation of the synovium. This inflammatory mediator may be detected in the early phase after ACL rupture.[1-3] The postoperative inflammatory reaction of ACL affects surgical timing and causes arthrofibrosis risk in early ACL reconstruction. Many studies showed, ACL reconstruction performed within 1 week showed a significantly increased arthrofibrosis compared to those which were delayed for 3 weeks.[4-6]

The pathophysiology of arthrofibrosis is thought to play a role in the inflammatory process occurring in the fat pad and synovium, which are followed by thickening of the joint capsule.[7] The presence of injury causes an inflammatory reaction which initiates the production of reactive oxygen species (ROS). The production of ROS will cause the release of mast cells and the proliferation of Fibroblast Growth Factor (FGF). TGF- β and growth factor of other platelets will initiate cascades that produce extracellular matrix proteins and protease inhibitors, as well as inhibition of proteolytic enzyme production. Autoregulation of TGF-β produces feedback mechanisms. Overexpression of TGF-β will cause tissue matrix and fibrosis deposition.[6]

Studies from Almekinders and colleagues show that patients undergoing reconstruction less than one month post-injury have limited knee joint movement either after surgery or one year after. The study by Passler also showed the occurrence of arthrofibrosis in 18% of patients who underwent surgery in the first seven days post-injury compared with 6% of patients when performed four weeks post-injury. Many literatures recommend that ACL reconstruction are performed on the third week after injury when inflammation subsided. Option for surgical intervention a few days after injury may be beneficial for professional or individual athletes who want to be able to return to their functional level as soon as possible. Whereas, the option for

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delayed intervention will be more attractive for those who do not have a time limit or want more time to prepare for the operation.

The biomarkers acting after ACL injuries include IL-6 and TNF-α.[2] In ACL injuries, synoviocytes, chondrocytes and other intra-articular tissues will be activated to produce inflammatory mediators such as IL-1β, IL-6, IL -8, IL-10 and tumor necrosis factor-α (TNF-α). Injuries performed on rabbit knee joints increase IL-1 β, IL-6, and IL-8 two to three times in 72 hours after injury, but will decrease within three weeks. While the study by Cuellar and colleagues who examined the cytokine profile after acute ACL injury showed cytokine that consistently increased, namely, the IL-6.[8]

Tea leaf extract is believed to be a potent antioxidant that can inhibit various diseases such as degenerative diseases and cancer. Green tea has been studied to reduce and inhibit pathologic processes of diseases mediated by inflammatory factors.[9] Green tea extracts in vivo and in vitro inhibitors may suppress an inflammatory process through inhibition of inflammatory mediators.[10] Reduction of inflammatory mediators such as IL -1β and TNF-α after administration of green tea extract showed a protective effect after ACL injury.[11] In a study by Ahmed, the concentration of IL-6 in serum coincides with damage occurred in the joints. EGCG in green tea extract also has an inhibitory effect on serum IL-6 levels and joint homogeneity.[12]

Green tea extract is expected to provide a benefit on ACL injury to reduce the inflammatory process which may accelerate the ACL reconstruction process and resulted in a good outcome.

MATERIAL AND METHODS

This research is an experimental study design using randomized post-test only control group design. This study uses 24 New Zealand white rabbits with female gender, weighing 2-3 kg as subjects. The rabbits were healthy with no defects. Rabbits were divided into six groups: three treatment groups given green tea extract and three control groups without the administration of green tea extract. ACL rupture is performed by transection of the right rabbit ACL. The 300 mg of green tea extract was dissolved and administered orally using 3cc syringe once a day. After transecting the ACL and administration of green tea extract, IL-6 levels and TNF-α expression were tested weekly. For the first week, serum IL-6 and TNF-α levels are tested three days after ACL injury on both the treatment and control group. For the second week, the treatment and control group was tested for serum IL-6 and TNF-α levels ten days after ACL rupture. For the third week, the treatment and control group was tested for serum IL-6 and TNF-α levels 17 days after ACL injury. Levels of serum IL-6 were examined using ELISA method, 2 cc of serum sample was taken from the blood vessels in rabbit ears using a 3 cc syringe and then was centrifuged. TNF-α expression was examined using a rabbit ACL tissue attached to the bone of a rabbit femur that has been sacrificed and fixed using a buffer solution.

RESULTS

Analysis of research included distribution of descriptive data of IL-6 and TNF-α. The data were analyzed statistically using SPSS for Windows version 22.0.

Analysis of descriptive data showed 24 research subjects distributed into treatment group with green tea extract and control group without green tea extract at first, second, and the third week, with each week consisting of 4 subjects or 12.50% of the total of all subjects.

The mean IL-6 concentration in treatment groups with green tea extract at first week was 92.023 ± 4.813, while in the treatment groups with green tea extract at second and third week were 20.073 ± 0.243 and 19.989 ± 0.532 pg / mL respectively. The mean IL-6 concentration in the control group without administration of green tea extract in weeks I, II and III were 1301.114 ± 522.454, 46.592 ± 30.665 and 20.117 ± 0.559 pg / mL respectively. For TNF-α expression, four samples (33.33%) in the treatment group with green tea extract at first week showed mild expression. However, the treatment group with green tea extract at second and third week also showed mild expression (33.3%). For the control group without administration of green tea extract as many as four samples (50%) at weeks I and II showed dense or solid expressions, while in the treatment group with green tea extract at Week III all showed moderate expression (100%).

Levels of TNF-α in the control group first and second week were highly expressed with a percentage of 50% of all samples with green tea extract extracts in first, second, and third week expressed mildly with a percentage of 33.3% of all samples. In the control group without green tea extract in third week 100% showed moderate expression.

The inferential statistical test used in this study is one-way ANOVA with post hoc LSD when the data is homogeneous, or with post-hoc, Tamhane's T2 when the data obtained is not homogeneous. Assessment of test results used 95% CI and p-value on the significant margin of 0.05. The research variables in the treatment and control group were

Table 1 Mean of IL-6 value on each treated group and control group

Variable	GROUP					
	Treated Group With Green Tea Extract 1st Week (n=4) (Mean ± SD)	Treated Group With Green Tea Extract 2nd Week (n=4) (Mean ± SD)	Treated Group With Green Tea Extract 3rd Week (n=4) (Mean ± SD)	Control Group Without Green Tea Extract 1st Week (n=4) (Mean ± SD)	Control Group Without Green Tea Extract 2nd Week (n=4) (Mean ± SD)	Control Group Without Green Tea Extract 3rd Week (n=4) (Mean ± SD)
IL-6 (pg/mL)	92.023 ± 4.813	20.073 ± 0.243	19.989 ± 0.532	1301.114 ± 522.454	46.592± 30.665	20.117 ± 0.559

Table 2 TNF-α expression percentage on each treated group and control group

Variable	Expression Category	Group						
		Treated Group With Green Tea Extract 1st Week (n=4) n (%)	Treated Group With Green Tea Extract 2nd Week (n=4) n (%)	Treated Group With Green Tea Extract 3rd Week (n=4) n (%)	Control Group Without Green Tea Extract 1st Week (n=4) n (%)	Control Group Without Green Tea Extract 2nd Week (n=4) n (%)	Control Group Without Green Tea Extract 3rd Week (n=4) n (%)	Total
TNF-α	R Dense	0 (0%)	0 (0%)	0 (0%)	4 (50%)	4 (50%)	0 (0%)	8 (33.3%)
	Moderate	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (100%)	4 (16.7%)
	Mild	4 (33.3%)	4 (33.3%)	4 (33.3%)	0 (0.0%)	0 (0%)	0 (0%)	12 (50%)
Total		4 (16.7%)	4 (16.7%)	4 (16.7%)	4 (16.7%)	4 (16.7%)	4 (16.7%)	24 (100%)

Table 3 Comparability study result between the post-test experimental variable of treated group and control group

Variable	Group							
	Treated Group With Green Tea Extract 1st Week (n=4) (Mean ± SD)	Treated Group With Green Tea Extract 2nd Week (n=4) (Mean ± SD)	Treated Group With Green Tea Extract 3rd Week (n=4) (Mean ± SD)	Control Group Without Green Tea Extract 1st Week (n=4) (Mean ± SD)	Control Group Without Green Tea Extract 2nd Week (n=4) (Mean ± SD)	Control Group Without Green Tea Extract 3rd Week (n=4) (Mean ± SD)	F	P Value
IL-6 (pg/mL)	92.023 ± 4.813	20.073 ± 0.243	19.989 ± 0.532	1301.114 ± 522.454	46.592± 30.665	20.117 ± 0.559	23.302	0.000

tested for normality. With the amount of data of 24 (n <50), the normality test used for IL-6 is Shapiro-Wilk test, while the homogeneity test of variance data is done using Levene's test. In this research, IL-6 data distribution is normal, the p> 0.05 and showed homogeneous variant data, where p <0.05.

For numerical variables, a test for significance was performed on two groups of data using One-Way ANOVA test for normally distributed data. To know the effect of each variable in the treatment and control group, the post-test mean of each group was compared.

The table above showed that IL-6 levels in all treatment groups were lower than in all control groups, and the mean difference between treatment and control groups was statistically significant with p = 0.000 (p <0.05). To know the mean difference of mean between the groups, a post hoc test is performed as follows.

The table above showed that in the treatment group with green tea extract administration at first week showed a lower level of IL-6 than the control group and compared with the treatment group at the second and third week which was statistically significant with p = 0.000 (p <0.05). However, the treated group with green tea extract at second week did not show any significant differences in IL-6 level as compared with the third-week group with p = 0.863 (p> 0.05). This result suggests that there was no significant difference in IL-6 levels after two weeks of administration of green tea extract.

In this research, TNF-α expression was an ordinal variable and categorically served. Thus non-parametric analytic was performed using Kruskal-Wallis.

The table above showed that TNF-α expression in the treatment group with green tea extract in the

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Table 4 Result of post hoc LSD test between research variables for treatment and control group

Variable	(I) Group	(J) Group	Mean Different	p	95% Confidence Interval	
					Lower Bound	Upper Bound
IL-6	Treated Group With Green Tea Extract 1st Week	Treated Group With Green Tea Extract 2nd Week	1254.521	0.000	937.102	1571.941
		Treated Group With Green Tea Extract 3rd Week	1280.997	0.000	963.577	1571.941
		Control Group Without Green Tea Extract 1st Week	1209.092	0.000	891.673	1526.512
		Control Group Without Green Tea Extract 2nd Week	1281.041	0.000	963.621	1598.460
		Control Group Without Green Tea Extract 3rd Week	1281.124	0.000	963.705	1598.543
	Treated Group With Green Tea Extract 2nd Week	Treated Group With Green Tea Extract 1st Week	-1254.521	0.000	-1571.941	-937.102
		Treated Group With Green Tea Extract 3rd Week	26.475	0.863	-290.944	343.894
		Control Group Without Green Tea Extract 1st Week	-45.429	0.767	-362.848	271.990
		Control Group Without Green Tea Extract 2nd Week	26.519	0.863	-290.900	343.938
		Control Group Without Green Tea Extract 3rd Week	26.602	0.862	-290.816	344.021
	Treated Group With Green Tea Extract 3rd Week	Treated Group With Green Tea Extract 1st Week	-1280.997	0.000	-1598.416	-963.621
		Treated Group With Green Tea Extract 2nd Week	-26.475	0.863	-343.894	290.944
		Control Group Without Green Tea Extract 1st Week	-71.904	0.640	-389.323	245.515
		Control Group Without Green Tea Extract 2nd Week	0.044	1.000	-317.375	317.463
		Control Group Without Green Tea Extract 3rd Week	0.127	0.999	-317.292	317.546

Table 5 Kruskal-Wallis study result between post-test data experimental variable of treated group and control group

Variable	Mean Rank						P-Value
	Treated Group With Green Tea Extract 1st Week (n=4) (Mean ± SD)	Treated Group With Green Tea Extract 2nd Week (n=4) (Mean ± SD)	Treated Group With Green Tea Extract 3rd Week (n=4) (Mean ± SD)	Control Group Without Green Tea Extract 1st Week (n=4) (Mean ± SD)	Control Group Without Green Tea Extract 2nd Week (n=4) (Mean ± SD)	Control Group Without Green Tea Extract 3rd Week (n=4) (Mean ± SD)	
TNF-α	10.5	5.0	4.0	17.7	20.5	17.2	0.001

first week showed a lower mean rank (10.5) than the control group without green tea extract (17.7) which was statistically significant with p-value = 0.0001 (p <0.05). Similarly, the treatment group with green tea extract at second and third week showed a lower mean rank (5.0 and 4.0) respectively than the control group without green tea extract (20.5 and 17.2) and is statistically significant with p-value = 0.001 (p <0.05). It showed a lower TNF-α expression in green tea extract administration. Following that, post hoc test was conducted to know the level of meaning between the treatment and control group.

The table above showed that the levels of TNF-α between the treatment groups and green tea extract at first and the second week was lower than the

Table 6 Post hoc study result between experimental variables of treated group and control group

Variable	(I) Group	(J) Group	Statistic Test	P
TNF-α	Treated Group With Green Tea Extract 1st Week	Treated Group With Green Tea Extract 2nd Week	0.000	1.000
		Treated Group With Green Tea Extract 3rd Week	0.000	1.000
		Control Group Without Green Tea Extract 1st Week	-14.000	0.002
		Control Group Without Green Tea Extract 2nd Week	-14.000	0.002
		Control Group Without Green Tea Extract 3rd Week	-8.000	0.080
	Treated Group With Green Tea Extract 2nd Week	Treated Group With Green Tea Extract 3rd Week	0.000	1.000
		Control Group Without Green Tea Extract 1st Week	14.000	0.002
		Control Group Without Green Tea Extract 2nd Week	-14.000	0.002
		Control Group Without Green Tea Extract 3rd Week	-8.000	0.080
	Treated Group With Green Tea Extract 3rd Week	Control Group Without Green Tea Extract 1st Week	14.000	0.002
		Control Group Without Green Tea Extract 2nd Week	14.000	0.002
		Control Group Without Green Tea Extract 3rd Week	-8.000	0.080

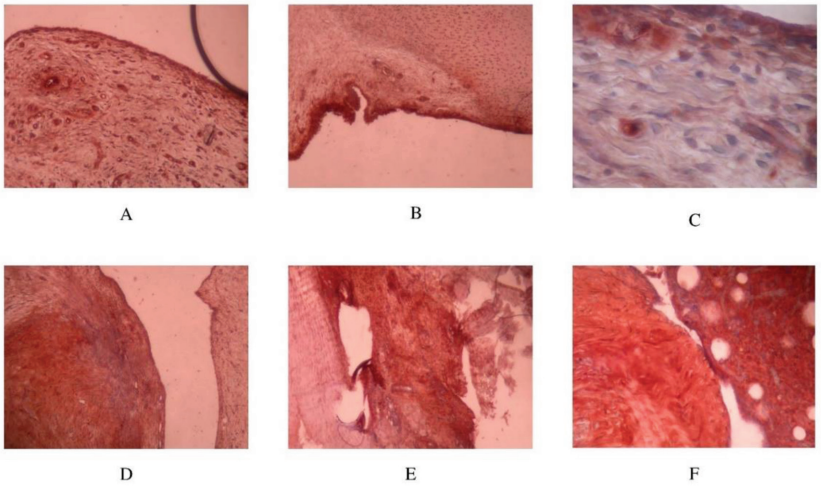

Figure 1 The histological result of TNF- α expression: A. Treatment group at first week; B. Treatment group at second week; C. Treatment group at third week; D. Control group at first week, E; Control group at second week; F. Control group at third week

control group and the difference was significant with p<0.05. However, the third-week treatment group did not show any significant differences with the control group (p-value = 0.08). Levels of TNF-α in the first week treated group did not show significant differences compared with treatment group second and third week with p>0.05.

DISCUSSION

The interpretation of the research data that has been processed and analyzed using statistics are in accordance with the hypothesis of the study. Next, the interpretation of the data will be discussed to determine the factors that influence the results of this study. The objective of this study is to determine the differences in serum IL-6 and TNF-α expression in ACL-treated rabbit group with green tea extract compared with no green tea extract.[24] rabbits were used as the samples.

In this study, a lower mean of IL-6 level was found in rabbits treated with green tea extract administration 300 mg/kgBW after ACL rupture and proved significantly different than control group without green tea extract administration with p = 0.000. Post ACL injury will increase levels of inflammatory factors such as IL-1β, bFGF, TGF-β, TNF-α, IL-6 and IL-8. The high levels of IL-6 secreted by chondrocytes in the first week following ACL injury were consistent with the Harkey study.[13] Cuellar's study also proved that peak concentrations of IL-6 synovial fluid levels occurred in 72 hour post-injury times as high as 2 to 3 fold.[8] The active ingredient of green tea extract, EGCG, is reported to inhibit the production of inflammatory mediators such as nitric oxide, prostaglandin E2, COX-2, inducible nitric oxide synthase (iNOS) and IL-8 in human chondrocytes in vitro.[10] Thus, from the research it can be postulated that with the administration of green tea extract, this inflammatory process can be suppressed especially in the first week after ACL rupture. This study used rabbit serum as a medium for IL-6 measurements, unlike other studies that measured synovial fluid. This study is also similar to the research conducted by Ahmed which proves that the content of EGCG in green tea extract has an inhibition effect on serum levels IL-6. Ahmed's research also states that high levels of IL-6 in serum coincidence with damage that occurs in joints.[12]

While post hoc test in this study showed significant differences in the levels of IL-6 after administration of green tea extract in the first week was proven to be statistically significantly different

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with a p<0.05. However, this post hoc test showed no significant difference after administration of green tea extract in the second and third weeks with p = 0.863 (p>0.05). In this study, serum IL-6 levels showed a tendency to decrease after the first week after the injury. This decreasing tendency is consistent with Cuellar's study of the peak IL-6 levels in synovial fluid occurring at 72 hours after ACL injury and will decline thereafter.[8] This is also similar to a study by Cameron and colleagues.[1] This cause the administration of the extract green tea in rabbits did not show statistically significant results after the first week although the mean lower yields on green tea extract in the first, second, and third week. This study differs from a study conducted by Batta and colleagues that showed high levels of IL-6 in synovial fluids up to 50 weeks.[3] High IL-6 levels in local synovial fluid and serum IL-6 levels may be affected by the systemic process and individual treatment.

In this study, there was a lower mean ranking of TNF-α expression in rabbits with administration of green tea extract 300 mg/kgBB during first, second, and third week after ACL rupture which proved to be significantly different from the control group without green tea extract with p = 0.001 (p <0.05). In the treatment group with administration of green tea extract, the distribution of TNF-α expression is only focal and multifocal. While in the control group not administered with green tea showed a diffuse picture on the ACL immunohistochemistry examination. This is consistent with research by Katiyar and colleagues showing the effect of polyphenols present in green tea extract given orally to reduce COX-2 and TNF-α in mice.[14] The study by Norris and colleagues also showed a protective effect of post-injection green tea ACL.[11] However, this study also denied the study by Ahmed stating that TNF-α levels remained unchanged by administration of green tea extracts.[12] This result could be due to the direct investigation of immunohistochemically damaged local ACL tissue via five field of view, unlike Ahmed's research that uses systemic fluid markers.

In the post hoc test the study, lower TNF-α expression between treatment groups with green tea extract in the first and second weeks was found in comparison with the control group and the difference was significant with p<0.05. Meanwhile, the third-week treatment group administered green tea extract with control group did not show statistically significant result with p>0.05. Levels of TNF-α in the first-week treatment group did not show a significant difference when compared with treatment group second and third week with p>0,05. In the control group not administered with green tea extract showed TNF-α expression that remained high in the first, second, and third week. This result is in contrast to Cameron's study showing that TNF-α serum varies and tends to be decreased after three weeks post-injury.1 However, this study is in line with research from Bigoni and colleagues showing constant levels of TNF-α levels in acute, or subacute (more than 15 days).[15]

CONCLUSION

Green tea extract in rabbits with ACL rupture resulted in lower IL-6 and TNF-α expression in the first, second, and third week than rabbits who were not administered green tea extract.

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