Original Research

Expression of toll like receptors before and after coronary angiography with isomolar contrast among those with and without coronary atherosclerosis

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Angiography is a safe technique for the detection of and treatment of cardiovascular diseases. However, the effects of the technique on the molecular response of the immune system are yet to be clarified. Toll like receptors (TLRs) are the important molecule participate in the innate immunity responses and induction of inflammation. This project was designed to explore the effects of angiography on the expression of TLR1, TLR2, TLR3 and TLR4. Fifty-five participants, including three separate groups (without artery stenosis, with one artery stenosis and more than one artery stenosis), were assessed in this project. TLR1, TLR2, TLR3 and TLR4 expression levels were evaluated in peripheral blood immune cells by measuring mRNA before and after angiography using Real-Time PCR techniques. mRNA levels of TLR1, TLR2 and TLR3 were significantly increased following angiography. Expression of TLR4 did not change after angiography. Other criteria also showed no correlation on TLR expression after angiography. TLR4 mRNA levels had a positive correlation with age in the participants without artery stenosis. Angiography may induce inflammation in subjects without artery stenosis via up-regulation of TLR1, 2 and 3 which may lead to cardiovascular diseases related complications.

Keywords

Artery stenosis; Angiography; Toll like receptor

1. Introduction

Cytokines are the main immune cell mediators and they play critical roles in several immune system related functions, including inflammation, angiogenesis/angiostasis [1]. Innate immune cells are induced to produce immune cell related molecules, including cytokines, via recognition of their ligands by pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) [2]. The immune cells use various extra-membrane and intra-cytoplasmic receptors, called pathogen recognition receptors (PRRs), to recognize PAMPs and DAMPs, and this mechanism represents the first immune response against foreign antigens [2]. Toll-like receptors (TLRs) are members of the PRRs that were discovered in the *drosophila* and called "Toll". Later, they were discovered in the mammalians and called TLRs because of their similarities to the Toll receptors discovered in *drosophila* [3]. TLRs play crucial roles in the recognition of the pathogen (PAMPs) and non-pathogen (DAMPs) related molecules and subsequent activation of immune cells [4]. Ten TLRs have been identified on the cell membrane and inside the cytoplasmic vesicles in the human immune cells and are numbered from TLR1 to TLR10 [3]. The molecules exert their activities after homo or hetero dimerization, for example, TLR1 and TLR2 make a TLR1/TLR2 dimmer that recognizes several PAMPs and DAMPs [5]. TLR3 and TLR4 make homodimers that recognize double strand RNAs (dsR-NAs) and lipopolysaccharides (LPS), respectively [6]. However, the TLRs have more ligands, for instance, TLR3 and 4 recognize internal DAMPs and play key roles in the pathogenesis of inflammatory-based diseases [6]. Therefore, it has been hypothesized that TLRs, including TLRs 1 to 4, may be considered as the main molecules participate in the induction of inflammation in the human.

X-ray is a part of angiography and may be associated with some side effects in patients undergoing angiography [7] and due to the fact that inflammation is a risk factor for the patients suffering from cardiovascular diseases, it has been hypothesized that angiography may be associated with some complications.

Based on the critical roles played by TLRs in the regulation of inflammation, their induction, as a response to angiography, can be considered as a risk factor for in patients with cardiovascular disorders. Thus, due to the pro-inflammatory roles played by TLR1, TLR2, TLR3 and TLR4 and the cor-

Table 1. Relative expression of TLR1, TLR2, TLR3 and TLR4, before and after angiography in the patients without artery stenosis, with one artery stenosis and more than one artery stenosis.

Target	Angiography	Without stenosis	With one artery stenosis	More than one artery stenosis	p value
TLR1	Before	0.67 ± 0.23	1.50 ± 0.81	1.44 ± 0.53	0.222
	After	2.19 ± 0.59	2.09 ± 1.43	1.20 ± 0.37	0.782
	p value	**0.042**	0.688	0.647	-
TLR2	Before	0.4 ± 0.14	0.80 ± 0.45	1.19 ± 0.43	0.363
	After	4.25 ± 1.5	0.99 ± 0.74	1.36 ± 0.86	0.088
	p value	**0.033**	0.755	0.867	-
TLR3	Before	0.35 ± 0.15	0.84 ± 0.36	1.32 ± 0.52	0.204
	After	3.28 ± 1.14	0.33 ± 0.13	1.00 ± 0.70	**0.016**
	p value	**0.032**	0.138	0.735	-
TLR4	Before	1.13 ± 0.88	0.71 ± 0.36	1.00 ± 0.40	0.860
	After	1.81 ± 0.68	0.73 ± 0.47	0.71 ± 0.38	0.366
	p value	0.585	0.975	0.633	-

Data analysis revealed that relative expressions of TLR1, TLR2, TLR3 were significantly increased after angiography in the patients without artery stenosis. Relative expression of TLR3 was significantly different among the groups after angiography.

Table 2. Relative expression of TLR1, TLR2, TLR3 and TLR4 in the female versus male in the groups without artery stenosis, with one artery stenosis and more than one artery stenosis.

Target	Gender	Without stenosis	p value	With one artery stenosis	p value	More than one artery stenosis	p value
TLR1	Female	0.68 ± 0.33	0.772	0.06 ± 0.04	0.550	0.74 ± 0.32	0.424
	Male	0.54 ± 0.31		1.21 ± 0.66		2.86 ± 1.35	
TLR2	Female	0.27 ± 0.08	0.444	0.01 ± 0.004	0.515	1.24 ± 1.04	0.944
	Male	0.50 ± 0.32		0.93 ± 0.51		1.17 ± 0.46	
TLR3	Female	0.30 ± 0.08	0.563	0.03 ± 0.01	0.454	1.53 ± 1.31	0.811
	Male	0.46 ± 0.30		0.84 ± 0.36		1.23 ± 0.55	
TLR4	Female	0.26 ± 0.09	0.271	0.02 ± 0.01	0.517	2.27 ± 1.01	0.221
	Male	2.29 ± 2.05		0.8 ± 0.4		0.67 ± 0.33	

Data analysis revealed that relative expression of TLR1, TLR2, TLR3 and TLR4 were not different between male and females in the groups before angiography.

relation that inflammation has with cardiovascular diseases, this project was designed to explore the affect of the angiography on the expression of these molecules.

2. Material and methods

2.1 Subjects

mRNA levels of TLR1, TLR2, TLR3 and TLR4 were evaluated in 55 subjects. Based on the angiography criteria and vessel obstructions the participants were divided into groups as follows: without artery stenosis (19 cases), with one artery stenosis (18 cases) and more than one artery stenosis (18 cases). The patients were further sorted on the following criteria: age, sex, diabetes, smoking, drugs, alcohol consumption and opium usage. Exclusion criteria included immune related diseases, such as autoimmunity, allergies, infections and kidney diseases. The participants without artery stenosis were considered as controls, because they did not suffer from artery stenosis.

Angiography criteria were determined by an expert cardiologist, and factors that were considered included the existence of ACS containing unstable angina, ST elevation myocardial infarction (STEMI), non-ST elevation myocardial infarction (NSTEMI), typical chest pain and positive exercise stress test (EST). Therefore, the patients were selected as those suspected of having or developing atherosclerosis.

Angiography was carried out by an expert MD cardiologist using the comparison of the damaged to normal vessels after local anesthesia and using 6 French sheath and judkins catheters (left and right catheters). To compare the damaged to normal vessels, the contrast media, Visipaque, was used by direct injection into the left and right coronary arteries, in multiple projections.

Before and 3 hours after angiography, the peripheral blood samples were collected in pre-treated coagulant agent tubes to separate total mRNA. Consent forms were filled out by the participants and Islamic Azad University, Sciences and Research Unit Ethical Committee confirmed the investigation protocol (code: IR.IAU.SRB.1398.168).

2.2 Total RNA extraction and cDNA synthesize

A total mRNA extraction kit from Karmania Pars Gene Company, Kerman, Iran, was used to purify mRNA from the peripheral blood immune cells according to the manufacture's guidelines. Briefly, 200 μL blood was added to 500
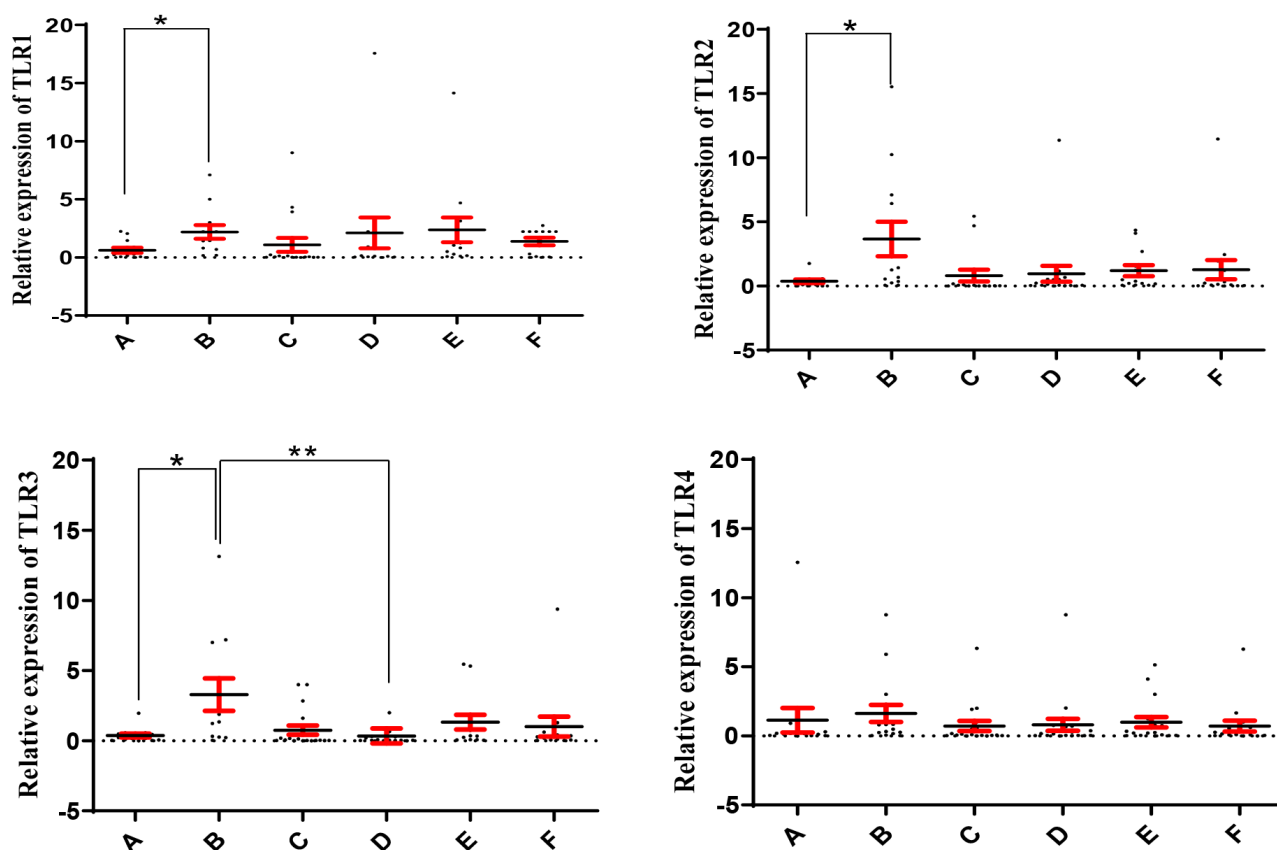
Fig. 1. Relative expression of TLR1, TLR2, TLR3 and TLR4 among the participants. Data analysis using paired t-test revealed that TLR1 ($p = 0.042$), TLR2 ($p = 0.033$) and TLR3 ($p = 0.032$) expression significantly increased after angiography when compared to before angiography in the participants without artery stenosis. Results showed that the relative expression of TLR3 was higher in the participants with one artery stenosis after angiography in comparison to the patients with one artery stenosis ($p = 0.014$). (A) Without artery stenosis before angiography. (B) Without artery stenosis after angiography. (C) With 1 artery stenosis before angiography. (D) With 1 artery stenosis after angiography. (E) With more than 1 artery stenosis before angiography and (F) With more than 1 artery stenosis after angiography.

*Data analysis within the groups regarding relative expression of TLRs before versus after angiography using Paired t-test.

**Data analysis among the groups after angiography regarding relative expression of TLRs. Accordingly, One way ANOVA test revealed that there were significant differences between groups after angiography ($p = 0.016$), and post-hoc Tukey test showed that the difference between B and D was significant ($p = 0.014$).

μL of lysis buffer and after 5 minutes incubation was centrifuged at 12000 RPM for 10 minutes. The supernatant was transferred to a new RNase free tube and 500 μL precipitation solution was added and incubated for 5 minutes at -20 °C. After that, the supernatant was added to a silica based column and centrifuged at 12000 RPM for 1 minute. The columns were washed by washing buffer twice and 30 μL of RNase free water was added and centrifuged at 12000 RPM for 1 minute. The pellet contained total mRNA, which was used for cDNA synthesize. Accordingly, cDNA was synthesized using a commercial kit from Karmania Pars Gene Company, Kerman, Iran, and based on the manufacture's instruction.

2.3 Real-Time PCR condition

Real-Time PCR was carried out using Real-Time PCR Assay commercial kits from Karmania Pars Gene Company, Kerman, Iran. According to the manufacture's instruction, the following Real-Time PCR program was defined for use in an Applied Biosystems Real-Time PCR machine (Step One Plus^{TM}): One step primary denature 95 °C for 10 minutes followed by 40 cycles of 95 °C for 30 seconds and 60 °C for 40 seconds. The results were calculated using the $2^{-\Delta\Delta Ct}$ formula.

2.4 Statistical analysis

To analysis the raw data, SPSS software version 20 was used and accordingly, the distribution of the data was evaluated using a One-Sample Kolmogorov-Smirnov test. Due to the normal distribution of the data, Paired-Samples t-test was used to analyze the relative expression of TLR1, TLR2, TLR3 and TLR4 before and after angiography within the groups. One way ANOVA was used to compare the relative expression of TLRs among the groups either before or after angiography separately. To analyse of the expression of the molecules in the male versus female within each group, before and after angiography, a student t-test was used. The

Table 3. Correlation among relative expression of TLR1, TLR2, TLR3 and TLR4 with x-doses and age in the without artery stenosis group.

			TLR1	TLR2	TLR3	TLR4	Age	X-dose
Before angiography	TLR1	Pearson correlation	1	**0.879**	**0.714**	−0.111	−0.119	-
		p value		**<0.001**	**0.006**	0.719	0.700	-
	TLR2	Pearson correlation	**0.879**	1	**0.978**	**0.655**	0.069	-
		p value	**<0.001**		**<0.001**	**0.021**	0.831	-
	TLR3	Pearson correlation	**0.714**	**0.978**	1	0.101	0.017	-
		p value	**0.006**	**<0.001**		0.731	0.953	-
	TLR4	Pearson correlation	−0.111	**0.655**	0.101	1	**0.660**	-
		p value	0.719	**0.021**	0.731		**0.010**	-
After angiography	TLR1	Pearson correlation	1	0.461	0.384	0.401	−0.171	0.069
		p value		0.132	0.273	0.197	0.594	0.831
	TLR2	Pearson correlation	0.461	1	**0.944**	**0.940**	−0.013	−0.172
		p value	0.132		**<0.001**	**<0.001**	0.968	0.573
	TLR3	Pearson correlation	0.384	**0.944**	1	**0.974**	0.163	−0.314
		p value	0.273	**<0.001**		**<0.001**	0.612	0.320
	TLR4	Pearson correlation	0.401	**0.940**	**0.974**	1	0.003	−0.091
		p value	0.197	**<0.001**	**<0.001**		0.992	0.737

correlation between the dose of X-rays (X-doses), age and relative expression of the TLRs, Pearson correlation test was used.

3. Results

Analysis of sex differences among the groups revealed that 10, 8 and 8 of the participants in the patients without artery stenosis, with one artery stenosis and more than one artery stenosis were female and 9, 10 and 10 were male, respectively ($p = 0.846$) suggesting the groups were evenly matched. Analysis of the age also showed that the patients without artery stenosis were 58.21 ± 2.46 years of age, with one artery stenosis were 58.66 ± 1.87 and with more than one artery stenosis had 60.06 ± 2.41 years ($p = 0.841$).

Data analysis revealed that relative expression of TLR1 were significantly increased ($p = 0.042$) in the without artery stenosis participants following angiography (0.67 ± 0.23 before and 2.19 ± 0.59 after angiography). TLR2 also increased after angiography and significantly increased from 0.4 ± 0.14 to 4.25 ± 1.5 ($p = 0.033$) in the without artery stenosis participants. TLR3 also were increased after angiography in the without artery stenosis participants. Accordingly, TLR3 relative expressions were 0.35 ± 0.15 before and 3.28 ± 1.14 after angiography in the patients without artery stenosis ($p = 0.032$). However, TLR4 relative expression was not changed after angiography in the without artery stenosis participants ($p = 0.585$) when compared to before angiography.

Data analysis in the patients with one artery stenosis revealed that TLR1 ($p = 0.688$), TLR2 ($p = 0.755$), TLR3 ($p = 0.138$) and TLR4 ($p = 0.975$) were not changed after angiography.

Relative expression of TLR1 ($p = 0.647$), TLR2 ($p = 0.867$), TLR3 ($p = 0.735$) and TLR4 ($p = 0.633$) were not also altered after angiography in the patients with more than one artery stenosis. Fig. 1 and Table 1 illustrate the raw data regarding relative expression of TLR1, TLR2, TLR3 and TLR4 in the participants before and after angiography.

As shown in Fig. 1, by using One way ANOVA, there were no significant differences among the groups before angiography regarding relative expression of TLR1 ($p = 0.222$), TLR2 ($p = 0.363$), TLR3 ($p = 0.204$) and TLR4 ($p = 0.860$). However, the relative expression of TLR3 ($p = 0.016$) was significantly different among the groups after angiography. Accordingly, post-hoc Tukey test revealed that the relative expression of TLR3 was significantly higher in the participants without artery stenosis than the patients with one artery stenosis ($p = 0.014$).

TLR1, TLR2, TLR3 and TLR4 expressions before angiography were not different between the males when compared to the females in the groups (Table 2).

Pearson correlation test showed that age had significant moderate relation with TLR4 relative expression before angiography in the without artery stenosis group. Although TLR1 relative expression had significant correlations with other TLRs in the group before angiography, it had no significant correlation with other TLRs after angiography. TLR2 relative expression had significant correlation with other TLRs when comparing before and after angiography, except TLR1 after angiography (Table 3).

The results demonstrated that age had significant moderate relation with TLR4 relative expression before angiography. Although TLR1 relative expression had significant correlations with other TLRs before angiography, it had not significant correlation with other TLRs after angiography. TLR2 relative expression had significant correlation with other TLRs at before and after angiography, except TLR1 at after angiography.

Table 4 revealed that all the TLRs had significant correlation with each other both before and after angiography in the groups with one artery stenosis.

Table 4. Correlation among relative expression of TLR1, TLR2, TLR3 and TLR4 with x-doses and age in the groups with one artery stenosis.

			TLR1	TLR2	TLR3	TLR4	Age	X-dose
Before angiography	TLR1	Pearson correlation	1	**0.811**	**0.913**	**0.899**	−0.302	-
		p value		**<0.001**	**<0.001**	**<0.001**	0.238	-
	TLR2	Pearson correlation	**0.811**	1	**0.952**	**0.902**	0.138	-
		p value	**<0.001**		**<0.001**	**<0.001**	0.623	-
	TLR3	Pearson correlation	**0.913**	**0.952**	1	**0.708**	−0.131	-
		p value	**<0.001**	**<0.001**		**0.001**	0.603	-
	TLR4	Pearson correlation	**0.899**	**0.902**	**0.708**	1	−0.086	-
		p value	**<0.001**	**<0.001**	**0.001**		0.735	-
After angiography	TLR1	Pearson correlation	1	**0.978**	**0.895**	**0.977**	0.431	−0.362
		p value		**<0.001**	**<0.001**	**<0.001**	0.142	0.225
	TLR2	Pearson correlation	**0.978**	1	**0.878**	**0.994**	0.291	−0.239
		p value	**<0.001**		**<0.001**	**<0.001**	0.241	0.34
	TLR3	Pearson correlation	**0.895**	**0.878**	1	**0.870**	0.354	−0.308
		p value	**<0.001**	**<0.001**		**<0.001**	0.178	0.245
	TLR4	Pearson correlation	**0.977**	**0.994**	**0.87**	1	0.300	−0.209
		p value	**<0.001**	**<0.001**	**<0.001**		0.198	0.378

The results demonstrated that TLRs had significant correlation with each others at both before and after angiography.

Although TLR1 had a significant correlation with relative expression of TLR2 before angiography in the patients with more than one artery stenosis, its expression had no significant correlation with TLR2 after angiography (Table 5).

There were no significant relations between X-dose and TLRs expression in all groups (Tables 3,4,5).

4. Discussion

TLRs are the most significant family of receptor in the innate immune cell that regulate cell function. The immune cells are suspected of causing artery stenosis via induction of inflammation. The potential roles played by TLRs in the induction of inflammation have been well documented, hence the molecules may significantly participate in the pathogenesis of atherosclerosis. Accordingly, it has been reported that either atherosclerosis or diabetes up-regulate expression of TLRs participate in the induction of some complications [8, 9]. For example, Getz *et al.* [8], showed that up-regulation of TLR4 on the macrophages leads to the uptake of aggregated LDL, which is a main mechanism contributing in foam cell formation. Thus, the factors that increase expression of TLRs may be considered as the risk factors for atherosclerosis. The results from this study revealed that angiography significantly increased the relative expression of TLR1, TLR2 and TLR3 in the participants without artery stenosis. However, the patients with one and more than one artery stenosis had no significant differences regarding the relative expressions of the TLRs. Additionally, the relative expressions of the TLRs were not different between groups before angiography. Therefore, it may be concluded that angiography is considered as a risk the induction of inflammation in the individuals who are not affected by artery stenosis in TLR1, TLR2 and TLR3 dependent manner. It has

been demonstrated that TLR1 and TLR2 make a heterodimer that is expressed on the cell surface and recognize DAMPs and PAMPs, and when stimulated can cause the activation of immune responses. Although previous investigations proposed that angiography can be associated with some side effects, including nausea [10], to the best of our knowledge, this is the first study to investigate the effects of angiography on the expression of TLRs. However, previous investigations proposed that TLR1 and TLR2 are expressed on the atherosclerotic plaques and significantly participates in the stability of the plaque [11–13]. Moreover, the pathologic roles played by TLR3 in the pathogenesis of atherosclerosis have been reported by some investigations [14, 15]. Therefore, it may be hypothesized that angiography may be considered as a risk factor for induction of inflammation in the individuals displaying cardiovascular disease symptoms without artery stenosis in a TLR1, TLR2 and TLR3 dependent manner. Furthermore, based on the fact that TLR3 induced by angiography was the unique molecule that was higher in the participants without artery stenosis in comparison to the other groups, it may be concluded that the molecules or its signaling pathway, TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent, may be targeted for prevention of atherosclerosis.

The results showed that there were no significant differences between the male and the females regarding the relative expressions of the TLRs in all groups. In contrast with our results; Koupenova *et al.* [16], reported that the females with cardiovascular risk factors had higher expression of TLRs, including TLR1, TLR2 and TLR3, when compared to the males. The numbers of the female participants were lower than male in the current study. Therefore, similar sample sizes of males and females may need to be further investigated in regards to the differences seen in TLR expression.

Table 2 shows that there is a significant positive relation

Table 5. Correlation among relative expression of TLR1, TLR2, TLR3 and TLR4 with x-doses and age in the groups with more than one artery stenosis.

			TLR1	TLR2	TLR3	TLR4	Age	X-dose
Before angiography	TLR1	Pearson correlation	1	**0.967**	**0.945**	0.154	0.029	-
		p value		**<0.001**	**<0.001**	0.615	0.928	-
	TLR2	Pearson correlation	**0.967**	1	**0.971**	**0.951**	−0.478	-
		p value	**<0.001**		**<0.001**	**<0.001**	0.137	-
	TLR3	Pearson correlation	**0.945**	**0.971**	1	**0.985**	−0.516	-
		p value	**<0.001**	**<0.001**		**<0.001**	0.104	-
	TLR4	Pearson correlation	0.154	**0.951**	**0.985**	1	−0.284	-
		p value	0.615	**<0.001**	**<0.001**		0.304	-
After angiography	TLR1	Pearson correlation	1	0.567	**0.668**	0.438	0.202	−0.422
		p value		0.054	**0.018**	0.154	0.576	0.172
	TLR2	Pearson correlation	0.567	1	**0.983**	**0.934**	0.081	0.021
		p value	0.054		**<0.001**	**<0.001**	0.793	0.942
	TLR3	Pearson correlation	**0.668**	**0.983**	1	**0.996**	0.052	0.028
		p value	**0.018**	**<0.001**		**<0.001**	0.879	0.927
	TLR4	Pearson correlation	0.438	**0.934**	**0.996**	1	0.007	0.047
		p value	0.154	**<0.001**	**<0.001**		0.981	0.874

The results demonstrated that TLRs had significant correlation with each others at both before and after angiography. Although TLR1 had significant correlation with relative expression of TLR2 before angiography, its expression had not significant correlation with TLR2 after angiography.

between age and the relative expression of TLR4 in the participants without artery stenosis. Although angiography had no effects on the relative expression of TLR4, the potential roles played by TLR4 in the pathogenesis of atherosclerosis have been documented previously [15]. Therefore, it seems that age increases the risk of atherosclerosis through elevation of TLR4. Table 2 also revealed that there was no significant correlation between TLR1 and TLR2 after angiography in the participants without artery stenosis. Although both TLRs were up-regulated following angiography in the group, the lack of correlation between them may be related to the fact that TLR2 can also make a heterodimer with TLR6. Thus, this project proposes to evaluate other TLRs, including TLR6, following angiography.

The results showed that there were not relations between X-doses and the relative expression of TLRs. Thus, it may be hypothesized that X-dose may induce expression of TLR1, TLR2 and TLR3 in the participants without artery stenosis, but its elevation cannot be associated with higher expression of the molecules.

5. Conclusions

Angiography may be considered as a risk factor for induction of inflammation in a TLR1, TLR2 and TLR3 dependent manner in the individuals without artery stenosis but suffering from cardiovascular diseases symptoms including the existence of ACS containing unstable angina, STEMI, NSTEMI, typical chest pain and positive EST. In another words, there is no evidence regarding the adverse effects of angiography on the cardiovascular diseases, however, due to the fact that TLRs can participate in the induction of atherosclerosis [8], and angiography can increase expression of the TLRs, hence it appears that angiography may increase the risk of atherosclerosis. However, this is the first investigation regarding the roles played by angiography on the expression of TLRs and more investigations are required to clarify any correlations and subsequent treatments that could be used to reduce the risk of disease post-angiography.

Author contributions

All authors have read and approved the manuscript, and ensure that this is the case. LZ performed the laboratory tests and wrote the manuscript draft. AE and MKA designed the project, analyzed data and wrote the manuscript. MS performed the angiography.

Conflict of interest

The authors declare no conflict of interest.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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