Breastfeeding by Human Milk Significantly Increases the Expression of TLR4, TNF- α , CCL2, and CCL3 in the Prepuce Tissue of Neonates

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Abstract

Introduction: Innate immunity significantly participates in the tissue repair process. It has been documented that breastfeeding may alter immune responses. Thus, this project was designed to evaluate the effects of breastfeeding on the levels of TLR1-4, TNF- α , TGF- β , CCL2, and CCL3 in the prepuce tissue of neonates.

Material and methods: This project was performed on the 90 samples (45 cases with breastfeeding and 45 cases without breastfeeding) of prepuce tissue of neonates. The tissues were homogenized and mRNA levels of TLR1-4 and protein levels of TNF- α , TGF- β , CCL2, and CCL3 were evaluated by Real-Time PCR and ELISA techniques, respectively.

Results: Protein levels of TNF- α , CCL2, and CCL3 and mRNA levels of TLR4 were significantly decreased in the cases without breastfeeding when compared to the neonates with breastfeeding. There was a significant negative correlation between duration of pregnancy and mRNA levels of TLR1 in the neonates without breastfeeding.

Conclusion: Due to the results, breastfeeding can modulate the expression of TLR4 and its related cytokines/chemokines to improve its wound healing and fight against pathogens.

Introduction

It has been demonstrated that immune system and its related molecules significantly participate in the angiogenesis and tissue repair (1). Innate immunity plays key roles in this process, and accordingly, its related receptors may be considered as the responsible molecules involved in the angiogenesis and tissue repair (2). Interactions between innate immune receptors with their ligands lead to activation of intracellular signaling and finally production of various ranges of molecules, such as cytokines, that are involved in the tissue repair process (1). Toll like receptors (TLRs) are the main innate immune receptors that play key roles in the activation of immune cells (3). TLRs express on the both cytoplasmic membrane and intracellular vesicles in hetero- or homodimeric structures(3). TLR1 and TLR2 make a heterodimer receptor, while TLR3 and TLR4 express as homodimeric molecules (4). TLR1/TLR2 heterodimer induces myeloid differentiation primary response protein 88 (MyD88) pathway, TLR3/TLR3 homodimer can activate toll-Interleukin 1 receptor (TIR)-domain-containing adapter-inducing interferon- β (TRIF) pathway and TLR4/TLR4 homodimer uses both MyD88 and TRIF pathways (4). Therefore, these molecules cover two major pathways that activate innate immune cells and tissue repair (4). Additionally, activation of the molecules can lead to production of some cytokines, such as tumor necrosis factor alpha (TNF- α), transforming growth factor-beta (TGF- β), CC ligand 2 (CCL2) and CCL3, which plays significant roles in the tissue repair (5–8). The environmental factors that affect the expression of these molecules which can affect the process of tissue repair.

Tissue repair process is an important process following the circumcision in the neonates (9). Due to the fact that TLRs and their related cytokines significantly participate in the tissue repair, the environmental

factors such as breastfeeding that affect the expression of these molecules may be associated with altered tissue repair following circumcision. Therefore, this project was designed to evaluate the effects of breastfeeding on the tissue levels of TLR1-4, TNF- α , TGF- β , CCL2 and CCL3 in the neonatal prepuce tissue.

Material And Methods

Subjects

This project was performed on the 90 samples (45 cases with breastfeeding and 45 cases without breastfeeding) of prepuce tissue of neonates. The neonates were referred to the Urology Clinic of the Rafsanjan University of Medical Sciences. The neonates were completely healthy with no suffering from any known genetic and infectious diseases. The subjects also were not under treatment with the drugs that affect immune system. The pregnancy periods were between 37 to 42 weeks. The pre and post term neonates were excluded from the study. The demographic data, including neonate age, weight, and pregnancy time and also maternal age were collected using a check list. The prepuce tissue of neonates was collected immediately after circumcision and preserved in the radioimmunoprecipitation assay buffer (RIPA buffer) and then kept at -20 °C. The tissues were homogenized using a homogenizer vehicle (Novin-Abzar, Iran) and the homogenized tissues were used for RNA extraction and cytokine/chemokine assays.

The protocol of the study was approved by the Ethical Committee of the Rafsanjan University of Medical Sciences, Rafsanjan, Iran (IR.RUMS.REC.1398.095).

RNA extraction

Total RNA was extracted using a commercial kit (KPG-TDNA) from Karmania Pars Gene, Kerman, Iran. Accordingly, the homogenized tissues were lysed by the two lysis solutions and then the precipitation buffer were added to chelate RNA and the components were added to the high absorbance column. After centrifuge, washing buffer was added and then the columns were centrifuged again. Finally, 50 μ L pre-warmed DNase/RNase free water were added to the column and centrifuged to achieve the purified RNA.

cDNA synthesis

To synthesize cDNA, a commercial kit (KPG-cDNA) from Karmania Pars Gene, Kerman, Iran, was used. Briefly, 15 μ L from master mix was mixed by 5 μ g purified RNA and then incubated at 40 °C for 60 minutes. To inactive the reverse transcriptase (RT), the mixture was incubated at 75 °C for 5 minutes.

Real-Time PCR condition

To evaluate RNA levels of TLR1 (KPG-TLR1R), TLR2 (KPG-TLR2R), TLR3 (KPG-TLR3R), TLR4 (KPG-TLR4R), and GAPDH (KPG-GAPDHR) commercial kits from Karmania Pars Gene, Kerman, Iran, were used. The kits contain all material that is needed for amplifications of the target molecules, including specific primers. Based on the instruction of the kits, 15 μ L from master mixes (TLR1-4 and beta-actin) were

mixed by 3 μ L cDNA and 2 μ L DNase/RNase free water. A Rotorgene Real-Time PCR vehicle was used to amplification of the targets using the following program: 95 °C for 5 minutes (1 cycle), 95 °C for 20 seconds and 60 °C for 20 seconds (35 cycles). The program was followed by performing a melting temperature from 55 to 95 °C. The results were calculated using $2^{-\Delta\Delta ct}$ formula.

Cytokine/Chemokine assays

TNF- α (KPG-HTNF), TGF- β (KPG-HTGF), CCL2 (KPG-HCL2), and CCL3 (KPG-HCL3) tissues levels were evaluated using enzyme linked immunosorbent assay (ELISA) commercial kits from Karmania Pars Gene, Kerman, Iran. Accordingly, 8 points serial dilutions of the homogenized tissues were prepared using RIPA buffer and tested by the kits. The appropriate dilution was considered to prepare the homogenized tissues and using in ELISA tests.

Statistical analysis

Raw data were evaluated regarding its normal distribution using SPSS software version 18. The differences between two groups were examined using the student t test. The correlations between the variables were explored using Pearson correlation test. The significant value was considered at 0.05.

Results

The results demonstrated that protein levels of CCL2 ($P > 0.001$), CCL3 ($P > 0.001$) and TNF- α ($P = 0.003$) and also TLR4 mRNA levels ($P = 0.005$) were significantly decreased in the prepuce tissue of neonates without breastfeeding when compared to the prepuce tissue of neonates with breastfeeding. However, protein levels of TGF- β ($P = 0.309$), and mRNA levels of TLR1 ($P = 0.339$), TLR2 ($P = 0.069$), and TLR3 ($P = 0.255$) were not different between two groups. Figure 1 illustrated the protein levels of CCL2, CCL3, TGF- β , and TNF- α as well as mRNA levels of TLR1, TLR2, TLR3, and TLR4 the prepuce tissue. Figure 1 and 2 illustrate the protein levels of the cytokines and mRNA levels of TLRs, respectively.

As it is illustrated in the Table 1, Pearson correlation test revealed that there was a significant negative correlation between duration of pregnancy and mRNA levels of TLR1 in the neonates without breastfeeding ($r: -0.518$, $P: 0.023$). However, there were not significant correlations between the variables in the cases with breastfeeding (Table 2).

Table 1

Correlation between expression levels of TLR1-4, TGF- β , TNF- α , CCL2, and CCL3 with age of mothers and neonates, duration of pregnancy and neonate weights in the neonates without breast feeding.

		Age (Mothers)	Age (Neonates)	Duration of pregnancy	Weight (Neonates)
TLR1	Pearson Correlation	-0.010	0.124	-0.518	0.094
	P value	0.966	0.612	0.023	0.702
TLR2	Pearson Correlation	0.009	-0.146	-0.170	-0.153
	P value	0.961	0.441	0.369	0.418
TLR3	Pearson Correlation	-0.134	0.209	0.100	0.170
	P value	0.522	0.315	0.635	0.417
TLR4	Pearson Correlation	-0.297	0.282	0.351	0.173
	P value	0.204	0.228	0.129	0.466
TNF	Pearson Correlation	-0.146	-0.303	-0.010	-0.313
	P value	0.441	0.103	0.959	0.092
TGF- β	Pearson Correlation	-0.319	0.353	0.072	0.232
	P value	0.086	0.056	0.705	0.217
CCL2	Pearson Correlation	-0.148	-0.358	0.022	-0.306
	P value	0.434	0.052	0.909	0.100
CCL3	Pearson Correlation	-0.156	-0.276	0.022	-0.266
	P value	0.410	0.139	0.910	0.156
Pearson correlation test revealed that there was a significant negative correlation between duration of pregnancy and TLR1 mRNA levels in the neonates without breastfeeding.					

Table 2

Correlation between expression levels of TLR1-4, TNF- α , TGF- β , CCL2, and CCL3 with age of mothers and neonates, duration of pregnancy and neonate weights in the neonates with breastfeeding.

		Age (Mothers)	Age (Neonates)	Duration of pregnancy	Weight (Neonates)
TLR1	Pearson Correlation	0.218	0.128	-0.166	0.262
	P value	0.264	0.517	0.399	0.177
TLR2	Pearson Correlation	0.314	0.170	-0.169	0.348
	P value	0.104	0.386	0.391	0.069
TLR3	Pearson Correlation	0.200	-0.228	0.073	-0.156
	P value	0.384	0.319	0.754	0.499
TLR4	Pearson Correlation	0.169	-0.095	-0.032	0.011
	P value	0.399	0.637	0.874	0.955
TNF- α	Pearson Correlation	-0.058	0.021	0.008	-0.209
	P value	0.768	0.914	0.967	0.286
TGF- β	Pearson Correlation	-0.284	-0.097	-0.016	0.047
	P value	0.143	0.624	0.936	0.812
CCL2	Pearson Correlation	0.206	0.151	-0.109	-0.011
	P value	0.292	0.444	0.580	0.957
CCL3	Pearson Correlation	0.271	0.344	-0.124	0.098
	P value	0.163	0.073	0.529	0.620
Pearson correlation test revealed that there were not significant correlations between the variables in the cases with breastfeeding.					

Discussion

It has been demonstrated that CCL2 and CCL3 play key roles in the angiogenesis and tissue repair (5–8). Accordingly, the factors affecting the expression of the molecules in the tissues can be associated with modulation in tissue repair. The results demonstrated that the protein levels of these chemokines were

significantly lower in the neonates without breastfeeding in comparison to the neonates with breastfeeding. Additionally, the protein levels of TNF- α were also declined in the neonates without breastfeeding. Previous research has proved that TNF- α plays critical roles in the early process of wound healing in skin (10). The results demonstrated that protein levels of CCL2, CCL3 and TNF- α were significantly decreased in the neonates without breastfeeding. In another word, it appears that breastfeeding can improve the expression of the molecules participate in the skin of the neonates, which may be associated with improved wound healing following circumcision. Additionally, mRNA levels of TLR4 were also decreased in the neonates without breastfeeding. Due to the fact that TLR4 uses both MYD88- and TRIF- dependent pathways (11), and based on the fact that the receptor is the major responsible molecule to induce the expression of the cytokines and chemokines (12), it may be hypothesized that down-regulation of TLR4 is a major cause of decreased expression of CCL2, CCL3, and TNF- α in the neonates without breastfeeding. To the best of our knowledge, there are no reports of the cytokine and TLRs expression in the prepuce following circumcision. Accordingly, further research may be needed to clarify the effect of breastfeeding on the innate immunity-related gene expression profile of skin in the neonates. However, Portou et al., by review of several investigations reported that TLRs play key roles in the skin wound healing through activation of innate immune-related gene expression (13). Additionally, Seo et al., revealed that TLR4 is a major molecule participate in the production of the pro-inflammatory cytokines in the skin (14). Therefore, it appears that downregulation of TLR4 in the non-breastfeeding neonates can be associated with impaired tissue repair. In another word, breastfeeding with breast milk can be considered as an important factor to modulate normal expression of innate immunity receptors and cytokines. In parallel with our results, He and colleagues reported that human milk can modulate TLR-mediated inflammation (15). Another investigation revealed that human casein alpha s1, a main component of human milk, induces the expression of proinflammatory cytokines in a TLR4 dependent manner (16). Xiao et al., also showed that oligosaccharides within human breast milk can promote immune tolerance through interaction with TLR4 (17).

Moreover, the results showed that there was a negative correlation between duration of pregnancy and the TLR1 expression. Our previous investigations demonstrated that prolonged pregnancy can be associated with altered serum levels of mothers and neonates (18). Thus, it seems that increased duration of pregnancy from 35 to 42 weeks can be associated with decreased the expression of an important receptor, TLR1, in the skin tissue, which is involved in the tissue repair (19).

Our results revealed that maternal and neonatal age did not correlate with the expression of TLRs and the mentioned cytokines. Bermudez and colleagues reported that TGF- β and TNF- α production by the prepuce fibroblasts was positively correlated with age (20). In contrast, another study by Iram et al. revealed that age is associated with decreased functions of TLR3 in the skin (21). Due to the fact that the study by Iram and colleagues was conducted on the fetal, neonatal and adult donors, and compared the groups, it seems that the results may not be comparable to ours obtained from neonates. However, based on our results, maternal and neonatal age does not appear to be able to affect the expression of TLR14 and CCL2, CCL3, TGF β and TNF α .

Declarations

Ethics approval and consent to participate

The work was approved by the local ethical review board.

Consent for publication

All authors gave consent for the publication.

Competing interests

Authors have no conflict of interest to declare.

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Authors' contributions

Shadi Behfar designed the experiments, supervise the project, writing the manuscript; **Alireza Nazari** designed and performed the experiments, data analysis, wrote the manuscript, supervised the project; **Aliakbar Yousefi-Ahmadipour** data analysis, wrote the manuscript; **Soheila Pourmasoumi** performed the experiments; **Ahmadreza Sayadi** con data analysis; Mohammad **Kazemi Arababadi** wrote the manuscript.

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Figures

Figure 1

TNF- α , TGF- β , CCL2, and CCL3 protein levels in the prepuces of the neonates breastfeeding with human milk (Controls) and the neonates feeding by non-human milk (Case). The figure shows that protein levels of TNF- α , CCL2, and CCL3 significantly lower in the cases when compared to controls. *P= 003, **P> 001, ***P> 001.

Figure 2

TLR1, TLR2, TLR3, and TLR4 mRNA levels in the prepuce of the neonates breastfeeding with human milk (Controls) and the neonates feeding by non-human milk (Case). The figure shows that mRNA levels of TLR4 significantly lower in the cases when compared to controls. *P= 005.