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Human Milk MicroRNAs in the Development of Infant's Immune System: A Systematic Review

Abstract – Exclusive breastfeeding practice is relatively low among children below six months of age, despite the countless advantages provided by human milk. Improving and enhancing the immune system of the infants are important as their immune system is not completely developed yet in the early life. Breast milk or human milk is relatively abundant with bioactive components and nutrients and offers a complete source of food to the infants. The bioactive components of human milk are believed to provide infants with great protection. Currently, researchers discover the roles of microRNAs in human milk of humans towards proper immune system development of the infants. This systematic review aims to access and collect data on the diverse immune-related microRNAs in the human milk and their immunoregulatory functions. To obtain related articles pertaining to the human milk microRNAs, a systematic search was conducted by using three relevant databases namely Science Direct, Scopus and PubMed using appropriate keywords for the papers published in English between 2010 and 2019. The included papers in this systematic review were appraised using Crowe Critical Appraisal Tool (CCAT). From 314 potential papers identified initially, five papers met the inclusion criteria for this review. All papers were of moderate to high quality. Discussion: This systematic review has provided valuable information on numerous immune-related microRNAs that can be found in human milk, including their immunological functions. The most abundantly expressed microRNAs include miR-146b, miR-148a, and miR-146a. The findings suggest that the microRNAs found in human milk along with their immunoregulatory functions were crucial in strengthening immune system of the infants.

Keywords **– Human milk, microRNA, infant, immune system, immunological functions**

1 INTRODUCTION

Infant's development is significantly foreseen as essential and crucial matters to many parents to ensure the child grows healthily as well as strong physically and mentally. To achieve this well-being, the best method to provide infants with complete nutrients is through breastfeeding [1]. Breastfeeding not only has health benefits to the infant but also towards the mother and World Health Organization (WHO) had recommended exclusive breastfeeding for the infants until the sixth month of age to obtain optimum growth, development, and health [2]. Exclusive breastfeeding can be defined as the infant who obtained human milk only without being introduced with liquids or solids except for oral rehydration solution, vitamins and medicines [2]. Even though more awareness campaigns were launched to educate the community especially, mothers, on the advantages of breastfeeding, the rates of exclusively breastfeed repeatedly decline from the suggested practice by WHO [1]. Globally, WHO reported that only 40% of the young child aged below six months are exclusively breastfed [3]. Moreover, in Malaysia, based on National Health and Morbidity Survey data in 2016, the activity of breastfeeding by mothers exclusively for the infants up to sixth month of age, not yet reach the Global Nutritional Targets 2025 which is 50% [1]. These phenomena are quite worrisome because human milk of the mothers is the main source of nutrients, especially to the newborns.

The reason behind suggested breastfeeding is due to the benefits of a mother's human milk that contained various biomolecules and nutrients that are essential to an infant's development. In fact, a study done had shown that transferring of immunity from mother to infant through human milk, offers a nurturing environment which assist in developing infant's immunologic defenses and infant's intestinal mucosa as well as to protect the infants from infections [4]. Thus, human milk indeed is very crucial to guide the healthy developments of the infant's immune system.

This study aims to systematically review the existing literatures on the unique microRNAs found in human milk that fulfils the criteria of the Crowe Critical Appraisal Tool (CCAT) and to systematically review the evidence presented by the literatures on the roles of microRNAs in human milk to the development of the infant's immune system.

2 METHODS

Selection Procedure

Several online databases used in this study include Science Direct, PubMed, and Scopus. The articles were searched according to the specific keywords such as microRNAs or miRNAs, human milk, infant, and immune system or immunity from the databases and the related articles were listed. These articles were screened for any duplications and the duplicators were removed from the list. The articles then were selected by title and abstract related to the study and the relevant articles were identified. The assessment for the eligibility and reliability of the articles were done based on the inclusion criteria and through the quality assessment. Then, all the articles were reviewed and articles that meet the inclusion criteria were selected.

Search Strategies

Medical Subject Headings (MeSH) terms as the additional keywords were listed in this study. MeSH terms are the selected official phrases to denote certain biomedical terms [5]. MeSH words included were “microRNAs/immunology” [MeSH], "milk, human/physiology" [MeSH], "infant/immunology" [MeSH], and "immune system/physiology" [MeSH].

Inclusion and Exclusion Criteria

Factors to be considered for the inclusion of articles as the sources in this study are, (1) the reports must be written in English within 10 years back, (2) the articles must consist specific keywords such as microRNAs or miRNAs, human milk, infant, and immune system or immunity, (3) and the articles can be from the qualitative studies, quantitative studies, in-vivo studies, and in-vitro studies. On the other hand, the articles will be excluded (1) if it is written in different languages, (2) if the articles were published before 2009, and (3) if the articles are dissertation, proceeding, chapter of the books, hand searched, or grey literature.

Quality Assessment

The selected articles as the references in this study were assessed for its quality using Crowe Critical Appraisal Tool (CCAT). CCAT is one of the critical appraisal tools to rate the research papers. CCAT is comprised of the CCAT User Guide and the CCAT Form [6]. Both the CCAT Form and CCAT User Guide must be used together to ensure the validity and eligibility of the research papers through the scores obtained [5].

Data Extraction

Full-text articles that had been selected following the methodology of PRISMA 2009 in this study were reviewed to obtain particular information relevant to the objectives of this study [7]. All of the included articles were read thoroughly and essential data regarding microRNAs found in human milk and their important functions were highlighted in the Results.

3 RESULTS

Search Results

The selection procedure for the articles to be included in this systematic review was shown in Figure 1. At the beginning of the search, a total of 314 studies were obtained according to the appropriate keywords from the database searching. After removing 69 duplications, the titles of 245 articles were diligently screened and as a result, 223 articles were removed. At this stage, the abstracts of 22 articles were screened and seven articles were removed because of the non-relevance to the aims of this research, and the abstracts were not really matched with the intended ones. The remaining 15 full-text articles were carefully read and reviewed according the inclusion and exclusion criteria. The exclusion of ten full-text articles was because of several reasons, whereby, six articles were review articles, three articles were chapter of the books and one article was opinion. In total, five full-text papers were included in this systematic review as these studies complied with the inclusion criteria set earlier.

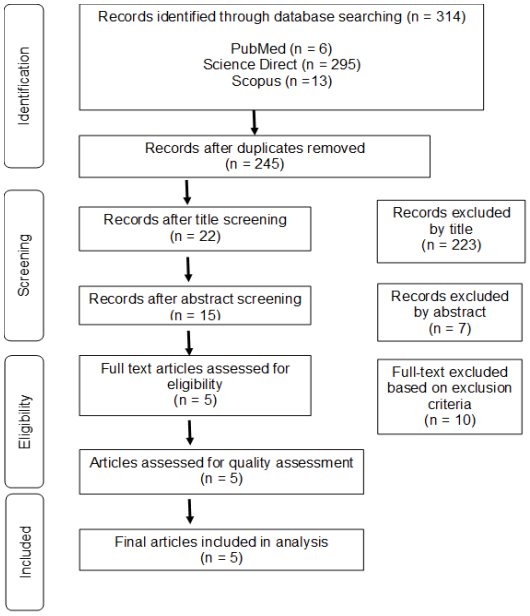

Figure 1: Flow diagram of selection procedure to obtain eligible full-text publications for the review.

Quality of the Included Studies

The quality of these five selected articles was assessed through eight categories such as title, abstract and text, introduction, design, sampling, data collection, ethical matters, results and discussion. The articles were considered as of medium to high quality based on the Crowe Critical Appraisal Tool (CCAT) score.

Study Characteristics

The main characteristics of the studies included were summarized in Table I. The five articles reviewed comprised 135 participants with 83 healthy mothers, 26 mothers with type 1 diabetes, and 26 healthy controls. The enrolment of mothers as subjects for all the studies was to obtain human milk samples for the detection of microRNAs expression. One study that had chosen mothers with type 1 diabetes was to compare the expression of microRNAs with healthy controls. Several methods were used to detect the expression of microRNAs in human milk such as miRNA microarray, small RNA sequencing technique and quantitative real-time polymerase chain reaction (qRT-PCR).

Table I Main characteristics of included studies in this systematic review.

Study No.	Reference	Country	Specimen	Collection Time	No. of Subjects	No. of Controls	Method	Participants
1	Kosaka et al., (2010)	Japan	Human milk	Within four days and 11 months postpartum	8	-	Microarray analysis and qRT-PCR	Mothers
2	Zhou et al., (2012)	China	Human milk	Two months postpartum	4	-	Small RNA sequencing	Mothers
3	Perri et al., (2018)	Italy	Colostrum and mature human milk	Postpartum	33	-	qRT-PCR	Mothers
4	Mirza et al., (2019)	Denmark	Human milk	Four weeks postpartum	26	26	Small RNA sequencing and qRT-PCR	Lactating mothers
5	Shiff et al., (2019)	Israel	Colostrum and 1-month milk	48 hours postpartum and 30 days postpartum	38	-	qRT-PCR	Mothers

Abbreviation: qRT-PCR: quantitative real-time polymerase chain reaction

Expression of MicroRNAs in Human milk

Numerous different immune-related microRNAs are found among the human milk samples. MicroRNAs such as miR-146b, [8, 9, 10], miR-148a [9, 11], and miR-146a [10, 11] are among immune-related microRNAs that are abundantly expressed. The expressions level of immune-related microRNAs found in the included studies were shown in Table II. There were two studies focusing on the expression of microRNAs between different stages of human milk including colostrum and mature human milk. The results revealed that the selected microRNAs (hsa-miR-21, hsa-miR-181a, hsa-miR-150, and hsa-miR-223) were comparably expressed by both colostrum and mature milk which suggested that the immune network of an infant was affected by these biological components at a very early stage [12]. In another study, the different expression of microRNAs between different periods of lactation was discovered when the immune-related microRNAs such as miR-181a, miR-17, miR-155, and miR-92 from the human milk in the first sixth month of age of infants presented with higher expression in comparison to these four microRNAs isolated from the human milk after the six months of age of infants [8].

Apart from that, there was one study that explored the difference in the expression of microRNAs between human milk of preterm infants' mothers and human milk of full-term infants' mothers. This study uncovered that miRNA-148a was highly expressed in the human milk of preterm infants' mothers compared to the human milk of full-term infants' mothers, while miRNA-320a was expressed more in the human milk of full-term infants' mothers than the human milk of preterm infants' mothers [11].

Stability of MicroRNAs in Harsh Environment

The stability of human milk microRNAs was studied by treating the microRNAs to several harsh and extreme environments. Human milk microRNAs were barely affected when treated with RNase, consequently demonstrated that the microRNAs can resist RNase digestion [8, 9]. Next, microRNAs in human milk were also stable in an acidic condition as there was only a slight difference in expression levels of human milk microRNAs between untreated and treated human milk in a low pH solution (pH 1) [8].

The microRNAs in human milk had been depicted to survive when subjected to the high temperature [9, 12], prolonged incubation [9], and frozen condition [8, 9, 12]. In high temperature, the recovery rate of microRNAs was about 70.3% (colostrum) and 67% (mature human milk) for all microRNAs that were expressed in this experiment [12]. The multiple freeze-thaw cycles illustrated that microRNAs were not really affected by this process as the relative expression levels of microRNAs showed only minor differences in every cycle [8, 9].

Regulatory Functions of Immune-Related MicroRNAs

One of the prominent functions of microRNAs is to have role in the regulation and maturation of T cell and B cell and these were responsible upon miR-155, miR-17, and miR-92 cluster [8]. miR-181a, which was discovered in two studies was proposed to contribute in the regulation of B cell differentiation and CD4+ T-cell selection [8]. Besides, miR-181a also contributed to the process of T cells thymic education and the myeloid cell lineage differentiation [12]. The latter role of miR-181a was demonstrated to be similar to miR-223. Quantitative analysis showed higher expression of miR-181a and miR-223, supporting the statement regarding preferential modulation on thymus and on networks of innate immune in addition to both microRNAs' roles stated previously [12]. Other regulatory roles of several immune-related microRNAs in human milk were presented in Table III.

Table II MicroRNAs and their expression level in the included studies.

A

Reference	Immune-Related MicroRNAs	Relative Expression Level (hsa-miR-181a/cel-miR-39)
Kosaka et al, (2010)	miR-155	1.0
	miR-181a	0.99
	miR-17	0.99
	miR-92	1.0

B

Reference	Immune-Related MicroRNAs	Total Reads of MicroRNAs (%)
Zhou et al., (2012)	miR-148a-3p	37.5
	miR-30b-5p	40.0
	let-7f-1-5p & -2-5p	45.0
	miR-146b-5p	49.0
	miR-29a-3p	50.0
	let-7a-2-5p & -3-5p	53.0
	miR-141-3p	55.0
	miR-182-5p	58.0
	miR-200a-3p	60.0
	miR-378-3p	62.3

C

Reference	Immune-Related MicroRNAs	Concentrations (ng/100ml)
Perri et al., (2018)	hsa-miR-21	11.96
	hsa-miR-181a	14.46
	hsa-miR-150	12.40
	hsa-miR-223	15.29

Table III Immune-related microRNAs with regulatory functions.

Immune-related microRNAs	Regulatory Functions	References
miR-155	• Involves in the regulation of T cell and B cell maturation and response of innate immunity.	Kosaka et al., 2010
miR-181a	• Regulates B cell differentiation and CD4+ T cell selection. • Play role in the process of T cells thymic education and in the myeloid cell lineage differentiation.	Kosaka et al., 2010; Perri et al., 2018
miR-181b	• Regulates B cell differentiation and CD4+ T cell selection.	Kosaka et al., 2010
miR-17 and miR-92 cluster	• The universal regulator to the development of B cell, T cell and monocyte.	Kosaka et al., 2010
miR-125b	• Functions as a negative regulator of tumor necrosis factor-α production, activation and sensitivity.	Kosaka et al., 2010
miR-146b	• Plays role as a negative regulator of the response of innate immunity.	Kosaka et al., 2010
miR-223	• Promotes proliferation and activation of neutrophil. • Play role in the process of T cells thymic education and in the myeloid cell lineage differentiation.	Kosaka et al., 2010; Perri et al., 2018)
let-7i	• Serves in the regulation of Toll-like receptor 4 expression in human cholangiocytes.	Kosaka et al., 2010
miRNA-320	• Involves in the gene expression regulation.	Shiff et al., 2019

Differentially Expressed MicroRNAs

The microRNAs expression was compared among the samples of human milk collected from healthy controls and mothers with type 1 diabetes. From nine differentially expressed microRNAs, six were up-regulated including hsa-miR-4497, hsa-miR-1246, hsa-miR-133a-3p, hsa-miR-3178, hsa-miR-1290, and hsa-miR-320d, whereas three were down-regulated including hsa-miR-518e-3p, hsa-miR-629–3p, and hsa-miR200c-5p [10]. This study explained that the target genes of these nine microRNAs were associated with cell cycle and pathways of the immune response. Based on the investigation for the possible effect on immune cell activity that was conducted on the most highly up-regulated human milk microRNAs of the type 1 diabetes mothers, hsa-miR-4497 and hsa-miR-3178 transfected cells increased the expression and secretion of TNF-α in the differentiated human monocytic THP-1 cells [10].

4 DISCUSSION

The data gathered in this study showed distinctive expression levels of microRNAs in different periods of lactation. The difference in the expression levels of microRNAs was due to changes in human milk's composition within feeds, diurnally, over lactation, and between mothers and populations [13]. In addition, the stage of lactation (colostrum versus mature milk) is one of the factors that had been demonstrated to have an impact on microRNA-mediated epigenetic regulation of immune responses as well as the development of the infants [14].

The result also explained the different expression levels of miRNA-148 and miRNA-320 in human milk of preterm infants' mothers and human milk of full-term infants' mothers. This is because it is suggested that the comparatively low level of miRNA-320 in the human milk of preterm infants' mothers allowed fatty acids synthesis for the short-term demands to assist the rapid growth of the preterm infant [11]. Moreover, the high level of miRNA-148 in the human milk of preterm infants' mothers is essential for the preterm infants because miRNA-148 apparently has roles that are crucial to the immune system of an infant as well as adipocyte numbers and functions that are still developing [11].

Based on the numerous existing studies, the stability of human milk microRNAs in infants are associated with the existence of extracellular vesicles. This is supported by the discovery of miR-181a and miR-17 in the isolation of CD63-positive exosome fraction that is detected through microarray analysis [8]. The microRNAs might be protected inside the exosomes or microvesicles which aid the microRNAs to survive in a harsh environment such as RNase treatment [8]. This finding also suggests that microRNAs are the genetic materials that are able to be transferred from a mother to an offspring [8]. The cumulative evidence in addition to the most current developments gathered has validated the bioaccessibility (withstand digestion) of milk microRNAs [15].

Apart from that, the resistance of microRNAs in human milk towards hot and cold conditions appeared to possess beneficial effects on the infants. Concerning low-birthweight neonates and infants that had been hospitalized, the frozen condition was dietetically crucial as the babies normally received freezer-stored human milk [8]. This statement was in concordance with a study that mentioned the pasteurized and freezer-stored colostrum or human milk that were often received by low-birthweight neonates and other hospitalized infants were beneficial as these conditions retain the immune regulatory function of microRNAs [12]. While fragile unprotected mammalian microRNAs could not resist certain harsh conditions, microRNAs of human milk are exceptional [15].

Next, the roles of microRNAs described in different studies are varied, whereby most studies provided information on the contribution of immune-related microRNAs towards the regulation and maturation of T cell and B cell. This finding is in parallel with one study which explained that microRNAs serve as the main role in the early differentiation and effector differentiation of B cells [16]. Besides, microRNAs also appear as the major regulators in the lineage induction pathways of T cells and possess an important function in the induction, function, and maintenance of the lineage of regulatory T cell [16]. MicroRNAs with distinctive functions were highlighted to identify the association between these roles to the immune system development of the infants. Referring to the immunoregulatory roles of microRNAs stated in this review, microRNAs possess a vital role in directing the appropriate behaviour of the immune system [16].

The differential expression of microRNAs was detected when comparing mothers with type 1 diabetes and healthy controls. This might be due to the greater variability of microRNAs to be likely dependent upon the characteristics of an individual including genetic, age, parity, diet as well as other environmental factors [17]. In another study, it is also suggested that the constitution of a mother and her living environments, for example, dietary intake and climate influenced their human milk microRNA's expression [8].

5 CONCLUSIONS

Overall, this study presents valuable information concerning microRNAs and their expression in human milk. The current review provides a useful

list of potential human milk microRNAs that had been recognized to produce positive effects that influenced the healthy formation of the infant's immune system. This synthesis of the existing study emphasizes on the immunological roles of various immune-related microRNAs in mothers' human milk.

6 CONFLICT OF INTERESTS

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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AUTHOR CONTRIBUTIONS

Zulkifli SNS wrote the main body of the paper. Ismail H and Zainuddin N provided feedback on the draft paper and approved the drafted manuscript. All authors read and approved the final manuscript.

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