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**Recommended Citation**  
Khudri, Ghaniyyatul; Sukmawati, Dewi; Barasila, Atikah Chalida; and Suryandari, Dwi Anita (2024)  
DOI: 10.7454/mss.v28i2.2234  
Available at: [https://scholarhub.ui.ac.id/science/vol28/iss2/7](https://scholarhub.ui.ac.id/science/vol28/iss2/7)

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Association between Macrophage’s Cell Number and Maternal Factors in Human Milk

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Received September 18, 2023 | Accepted June 5, 2024

Abstract

Infant nutrition and immune protection require the consumption of human milk, with macrophages mainly contributing to immune defense and infant development. This study was performed to investigate the correlation between macrophage cell numbers in human milk and various maternal factors. Thirteen human milk samples were collected from lactating mothers and were evaluated for macrophage cell numbers using a hemacytometer. The recorded questionnaire containing several questions, including age, multivitamin consumption, coronavirus disease 2019 vaccination status, allergy history, and lactation duration, was used to determine the maternal factors. Pearson’s correlation and Mann-Whitney test were employed in statistical analysis, with p-values < 0.05 considered significant. The macrophage cell number reached 54.236 ± 7.456 cells per mL. Notably, a significant correlation was observed between maternal allergy history and the number of macrophage cells in human milk (p = 0.049). Meanwhile, no statistically significant associations were detected among multivitamin consumption (p = 0.833), vaccination status (p = 0.923), and lactation duration (p = 0.236). This study emphasizes the effect of maternal characteristics on the composition and immune properties of human milk. The specific mechanisms underlying these correlations and their potential influence on infant health and development must be explored in future research.

Keywords: human milk, macrophages, maternal factor

Introduction

Human milk contains crucial nourishment, growth stimulants, and immunological constituents that support the ideal growth and development of newborns [1, 2]. Macrophages belong to immune factors present in human milk, and they contribute to the immune defense and protection of the infant [3, 4]. Macrophages perform phagocytosis, antigen presentation, and cytokine production and thus play a vital role in the modulation of immune response in infants [5, 6]. Human milk macrophages protect breastfed infants against microbial infections. These immune cells also contribute to the innate immune response by phagocytosing and eliminating pathogens present in the gastrointestinal tract, respiratory system, and other mucosal surfaces [7–9].

Emerging evidence suggests the considerable influence posed by maternal factors on the composition and function of human milk macrophages. Maternal age shows an association with variations in macrophage cytokine production, with older mothers potentially exhibiting various macrophage profiles compared with younger ones. Maternal nutrition, which includes the consumption of specific nutrients and supplements, also participates in the modulation of macrophage function [10]. Variations in human milk macrophage populations may also be attributed to vaccination status and lactation [11]. In the latest pandemic, an increasing number of lactating mothers received coronavirus disease 2019 (COVID-19) immunization. Althoe et al. (2022) presented evidence on the presence of anti-severe acute respiratory syndrome coronavirus 2-specific Immunoglobulin A, Immunoglobulin M, Immunoglobulin
G, and T cells in human milk [12]. Yang et al. (2020) demonstrated the notable increase in the M1 macrophage population after BCG vaccination. This finding implies a potential mechanism for the establishment of long-lasting protective immunity, which may offer insights into the immune response triggered by COVID-19 vaccination [13]. Passive immunization through breastfeeding evokes cellular response involving macrophage which can be highly boosted. These findings reinforce the importance of completing the vaccination of pregnant and lactating women to protect themselves and their infants from the severe manifestations of COVID-19. Comprehending the effect of these maternal factors on human milk macrophages can offer valuable insights into the optimization of breastfeeding practices and improvement of the immune protection provided to breastfed infants. The relationship between maternal factors and macrophage population in human milk must be understood to unravel the complex dynamics of maternal–infant immune interactions [7].

Therefore, this study aimed to investigate the macrophage cell number in human milk and explore the correlation between maternal factors and the presence of macrophages. We hypothesized the positive influence of maternal factors, such as maternal age, multivitamin consumption, COVID-19 vaccination status, allergy history, and breastfeeding duration, on the macrophage cell number in human milk.

Methods

This cross-sectional study received approval from the Ethical Committee of the Faculty of Medicine, Universitas Indonesia (Ket-434/UN2.F1/ETIK/PPM.0 02/2023). All procedures were conducted in accordance with the ethical guidelines set forth by the relevant human experimentation committee (at the institutional and national levels) and complied with the principles outlined in the 1964 Helsinki Declaration and its subsequent revisions.

The procedures were performed at the Oral Biology Laboratory, Faculty of Dentistry, Universitas Indonesia. Human milk was donated by healthy lactating women with infants aged within 6 months. The mothers had a maternal age range of 25–35 years and reported no history of antibiotic use, smoking, or alcohol consumption during breastfeeding. All participants are members of the Kampung Melayu District Primary Health Care outpatient clinic and were fully informed about the study. They submitted a signed documentation representing their informed consent before their inclusion in the study.

Milk samples were collected from thirteen lactating mothers in accordance with standardized procedures. The participants were requested to wash their nipples with soap and water before milk collection [14, 15]. Sterilized electric pumps were used in human milk collection. The collected samples were stored in a cooler box, transported at appropriate temperatures (approximately 4 °C), and directly analyzed in the laboratory within less than 2 h. A questionnaire was used to obtain data on maternal factors, including maternal age, multivitamin consumption, COVID-19 vaccination status, allergy history, and the breastfeeding duration.

We collected 20 mL human milk from the donor subjects and transported them in cooler bags for preservation at the temperature at 4 °C. Isolation of macrophages was performed in accordance with the modified methods of Silva et al. [16] and Saito et al. [17], which are based on the gradient centrifugation principle. The pellet was resuspended in complete Roswell Park Memorial Institute (RPMI) medium. Cell counting was conducted under an inverted microscope (Carl Zeiss) using a hemocytometer. Trypan blue exclusion assay was performed to assess the viability of isolated cells. The viable cells included those that did not stain blue, and the total number was considered representative of macrophage cell numbers.

GraphPad Prism 9.7.1 software was used in statistical analysis, and Pearson’s correlation and Mann–Whitney test were utilized to assess the correlation between maternal factors and the macrophage cell number. A significance level of $p < 0.05$ indicated statistical significance.

Results

After the application of the exclusion criteria, this study included 13 participants. Table 1 shows the maternal characteristics of the subjects, including the identified maternal factors, and the human milk macrophage cell number. The morphology of human milk macrophage after overnight culture can be seen in Figure 1.

Macrophages analysis revealed a significant cell number of 54,236 ± 7,456 cells per ml (mean ± standard error of the mean). In this study, mothers who had consumed supplements with a coefficient correlation showed a slightly higher number of macrophage cells in their milk ($r = 0.90$; Figure 2a). Nevertheless, in terms of correlation, the observed difference showed no statistical significance, as indicated by a $p$-value of 0.083 (Figure 2a).

An additional assessment of the maternal COVID-19 vaccination status during the last 12 months was performed to explore its potential association with the macrophage cell number in human milk. The initial questionnaire was not focused on determining COVID-19 vaccination status. However, the findings indicated homogeneity, which reveals that vaccinated mothers only received the COVID-19 vaccine. Further examination of maternal COVID-19 vaccination status...
Table 1. Maternal Characteristic

<table>
<thead>
<tr>
<th>Maternal Characteristic</th>
<th>Frequencies (%)</th>
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<tbody>
<tr>
<td>Maternal age (years)</td>
<td>29.00 ± 2.04*</td>
</tr>
<tr>
<td>Maternal multivitamin consumption†</td>
<td>5 (38.5%)</td>
</tr>
<tr>
<td>Maternal COVID-19 vaccination status‡</td>
<td>11 (84.6%)</td>
</tr>
<tr>
<td>Maternal allergy history</td>
<td>4 (30.8%)</td>
</tr>
<tr>
<td>Breastfeeding duration</td>
<td></td>
</tr>
<tr>
<td>14–30 days</td>
<td>4 (30.8%)</td>
</tr>
<tr>
<td>1–3 months</td>
<td>6 (46.1%)</td>
</tr>
<tr>
<td>4–6 months</td>
<td>3 (23.1%)</td>
</tr>
</tbody>
</table>

* mean ± standard error of the mean
† Folic acid, zinc, and calcium
‡ COVID 19 booster vaccine

Figure 1. Macrophages Cultured from Isolated Human Milk Monocytes using Roswell Park Memorial Institute (RPMI) Medium

was aimed at investigation of its potential correlation with the macrophage cell number in human milk. The analysis results suggest the slightly higher median count of macrophage cells than that in mothers without recent COVID-19 vaccination records (Figure 2b). However, the correlation did not achieve statistical significance (p = 0.923).

Similarly, no statistically significant correlation was observed in breastfeeding duration, which was denoted as the number of days from lactation initiation until sample collection (Figure 3). Meanwhile, a statistically significant and strong positive correlation was detected between maternal history of allergies and the number of macrophage cells in human milk (p = 0.049, r = 1.0) (Figure 2c).
Discussion

Human milk/breast milk composition shows dynamics following the status/condition of the mother and baby. Recent studies on human milk revealed live milk cellular components that are crucial for infant development. Such cellular components include cellular immune cells, including leucocytes and macrophages [8]. Macrophages can be found in all tissues, including human milk, and potential pathogens were monitored through amoeboid movement. In addition to phagocytosis, macrophages contribute to innate immunity and stimulate adaptive immunity through recruitment of other immune cells such as lymphocytes. They also serve as antigen-presenting cell to T cells. Dysfunctional or low-level macrophages in humans can lead to severe diseases, which results in frequent infections. Thus, macrophage is crucial for human immunity status [18]. This study analyzed the relationship between maternal characteristics, such as age, supplement consumption history, allergy history, vaccination history, breastfeeding duration, and the number of macrophages per milliliter of human milk (cells/mL).

According to the current research findings, the macrophage cell number in human milk during 1–6 months of breastfeeding was higher compared with those of previous studies by Zheng et al., where the macrophage number (cells/mL) during the first 1–11 months of breastfeeding reached 11,000 ± 5,000 [8]. The difference observed in this study implies the possible decrease in the macrophage content in human milk with the increase in breastfeeding duration of up to 11 months [19]. This research also presented data on the population of human milk macrophage cells and examined their association with lactation duration. Figure 3 indicates an increased trend in the total cell numbers with prolonged lactation. However, no statistically significant association was observed (p = 0.236), as supported by a weak positive correlation coefficient (r = 0.353). This observation contrasts with those of Zheng et al. and LeMaster et al. [8, 19], and this discrepancy can be attributed to the variation in sample distribution in terms of breastfeeding duration. In the present study, the breastfeeding duration within samples mostly (77%) lasted within 3 months, which indicated a high cell number. This sample homogeneity, as opposed to the previous study findings.
A moderate positive correlation was identified between the number of macrophages per milliliter of human milk and supplement consumption ($p = 0.083$). These results suggest the modest influence of maternal supplement consumption on the macrophage cell number present in human milk, albeit without reaching a statistically significant threshold. To our knowledge, no prior study compared the history of maternal supplement consumption with the quantity of macrophages in human milk. A response to a thorough inquiry and an extensive literature review revealed zinc as a key ingredient in most of the supplements consumed by the participants. A potential rationale for this finding can be linked to the inclusion of zinc in supplements. Zinc plays a critical role in the regulation and preservation of various physiological processes in macrophages, including the polarization of macrophages derived from human monocytic leukemia cell line (THP-1 cell line) [20]. Moreover, this element can augment the function of macrophages as immune-modulatory cells not only in human milk but also in various anatomical regions [20], [21, 22].

Furthermore, no significant correlation was observed between the macrophage cell number and maternal COVID-19 vaccination status. Regardless, Figure 2b shows a weak negative correlation, which implies the lower milk macrophage number of mothers who were vaccinated compared with those who did not receive vaccination. Although no prior study has been conducted on human milk macrophage number after vaccination, Alhoe et al. (2022) discovered another immune cell and specific antibody in human milk after the acquired COVID-19 vaccination [12].

A statistically significant negative correlation was observed between maternal allergy history and the number of macrophage cells in mother's milk ($p = 0.049$). Although no prior studies have explored the connection between maternal allergy history and the macrophage cell number in human milk, this discovery aligns with that of previous research indicating that maternal atopy can affect the immune composition of human milk [23].

Our findings provide valuable insights into the factors that influence macrophage cell number in human milk. Allergy history contributes to the regulation of the composition and the number of macrophages in human milk, whereas maternal COVID-19 vaccination, supplementation, and breastfeeding duration may also participate in the human milk macrophage population. Further research involving a larger sample should be conducted to clarify the potential influence of maternal characteristics on the quantity of macrophage cells in human milk. Comprehension of the mechanisms involved in this relationship and its implications for the immune function and protection of infants receiving human milk presents a challenge.

**Conclusion**

This study revealed a moderate negative correlation between maternal factors, such as maternal allergy history, COVID-19 vaccination status, lactation duration, and the macrophage cell count in human milk. Conversely, a positive correlation was observed with maternal supplement consumption. These findings underscore the influence of maternal factors in shaping the immune-related composition of human milk and shed light on the intricate dynamics of maternal–infant immune interactions. Further investigations in the future should focus on determining the underlying mechanisms and exploring potential implications for infant health and development.

**Acknowledgements**

This study is supported by the National Research and Innovation Agency of the Republic of Indonesia (BRIN - RIIM Batch-2 2022) and the Institute of Education Fund Management (Lembaga Pengelola Dana Pendidikan ~ LPDP) research grants to DS.

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