ORIGINAL

Effect of Human Breast Milk on ZO-1 and Occludin Expression in Caco-2 Cells Under Basal Conditions

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Running Title: Breast Milk and Intestinal Barrier

Abstract

Background: Necrotizing enterocolitis (NEC) is a serious inflammatory gastrointestinal condition affecting premature infants. Breast milk reduces NEC risk and contains bioactive components that may support intestinal epithelial barrier integrity. This study investigated whether pooled human milk alters the expression of ZO-1 (TJP1) and OCLN in intestinal epithelial cells.
Methods: In this in vitro experimental study, Caco-2 cells were treated for 48 hours with 0%, 1%, or 5% (v/v) pooled human milk in low-serum medium (0.5% FBS). Gene expression was assessed by real-time PCR, and protein levels were evaluated by Western blot.
Results: No statistically significant changes in ZO-1 or OCLN mRNA or protein levels were detected between treated and control groups ($P > 0.05$).
Conclusion: Under these experimental conditions, no statistically significant changes in total ZO-1 or OCLN mRNA or protein levels were detected after 48 hours of treatment with 1% or 5% pooled human milk. These findings do not exclude potential effects on tight-junction localization, assembly, or barrier function. Further studies with larger numbers of independent experiments and functional barrier assessments are warranted.

Keywords: Breast milk, Zonula Occludens-1, Tight junctions, Intestinal barrier, Necrotizing enterocolitis

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Zaremobini F, et al.

Introduction

Necrotizing enterocolitis (NEC) is a severe illness that leads to intestinal inflammation in extremely premature infants. This condition can harm the intestinal lining, weaken the body's immune system, enable harmful bacteria to invade the blood, and potentially lead to intestinal perforations, infections, and fatality [1]. Despite advancements in neonatal care, NEC remains a significant contributor to mortality and long-term complications in high-risk infants [2].

Breastfeeding is the most efficient way to lower the risk of NEC [3]. Human breast milk contains various active components such as growth factors, cytokines, antibodies, oligosaccharides, and lactoferrin [4].

Human breast milk can protect a baby's intestines by preserving the strength and integrity of the intestinal cell lining [5]. Tight junctions are responsible for maintaining the protective barrier of the intestine by controlling the movement of substances between cells and blocking harmful bacteria and antigens from crossing the gut lining [6]. Zonula Occludens 1 (ZO-1), also called Tight Junction Protein 1 (TJP1), is an important protein that helps connect transmembrane proteins in tight junctions to the actin cytoskeleton [7]. This protein plays a vital role in building and preserving the integrity of the tight junction barrier [8]. ZO-1 (TJP1) is a key tight-junction protein involved in intestinal epithelial barrier regulation. Although altered ZO-1 expression has been associated with

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impaired barrier function, its role in intestinal barrier maintenance appears more complex than previously thought [9]. Occludin (OCLN) is an important transmembrane tight-junction protein that contributes to epithelial barrier integrity and regulates paracellular permeability [10]. Higher OCLN expression has also been associated with improved epithelial barrier integrity, supporting its role in maintaining intestinal homeostasis [11].

While it is widely recognized that human breast milk offers protection against NEC, the precise molecular mechanisms that maintain the integrity of the epithelial barrier are not fully known. Specifically, there is a lack of information on how whole human breast milk directly affects the expression of genes related to tight junctions, like ZO-1 and OCLN, in epithelial cell models. Exploring these mechanisms further could help in understanding how breast milk supports the development of the intestinal barrier and guards against NEC.

Hence, the current research sought to examine how various levels of human breast milk impact the activity of the ZO-1 and OCLN genes in cultured epithelial cells. The assumption was that breast milk could boost the expression of these genes related to barriers, thus reinforcing epithelial strength and offering a better understanding of its protective function against NEC.

Materials and Methods

Human Breast Milk Preparation and Ethical Considerations

The experiments were performed using a commercially purchased human intestinal epithelial cell line and de-identified pooled human breast milk obtained from a certified human milk bank with documented donor consent and ethics approval. The pooled milk was centrifuged at 10,000 × g for 15 minutes at 4°C to separate the lipid fraction and remove cellular debris. The resulting skim milk was carefully collected, filtered through a sterile 0.22-µm membrane filter, aliquoted, and stored at -80°C until use. Aliquots were thawed only once before cell treatment, and the same pooled preparation was used for all experimental conditions. The research team received de-identified samples and had no direct contact with the donors.

Cell Culture

The Caco-2 cell line (human colorectal adenocarcinoma; ATCC® HTB-37™) was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were authenticated by the supplier and tested negative for mycoplasma before use. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) with high glucose, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 1% penicillin-streptomycin solution. The growth medium was refreshed every other day, and the cells were maintained under standard incubation conditions (37°C, 5% CO2, humidified atmosphere). Treatment experiments were carried out when the cell monolayers reached approximately 80% confluence. Cells between passages 25 and 35 were selected to minimize variability due to prolonged culture. Three independent cell culture experiments were performed using aliquots from the same pooled breast-milk preparation.

Experimental Groups and Treatments

For each independent experiment, cells were seeded in a single 9-well plate, with each treatment group (0%, 1%, and 5% milk) assigned three wells. Thus, all three groups were represented within the same plate in every experiment, so that plate-level variation (e.g., seeding batch and incubation conditions) was balanced across groups. The wells assigned to each group were distributed across the plate; however, treatment allocation was not formally randomized, and well positions were not systematically balanced. All cells were maintained for 48 hours in low-serum DMEM containing 0.5% FBS, supplemented as follows: the control group received no milk (0%), the 1% milk group received 1% (v/v) pooled human milk, and the 5% milk group received 5% (v/v) pooled human milk.

RNA Extraction and Quantitative Real-Time PCR

Following a 48-hour incubation period, RNA was isolated from Caco-2 cells using TRIzol® reagent (Invitrogen, Cat. No. 15596018). RNA integrity was assessed by agarose gel electrophoresis; all samples had A260/A280 ratios of 1.8–2.0 and clear 28S/18S rRNA bands. Next, 1 µg of RNA was reverse-transcribed into complementary DNA (cDNA) using oligo(dT) primers and M-MLV reverse transcriptase (Invitrogen, Cat. No. 28025013).

Transcript levels were quantified using a CFX96™ Real-Time PCR Detection System (Bio-Rad) with SYBR® Green Master Mix (Bio-Rad, Cat. No. 1708880). Primer sequences for TJP1, OCLN, and GAPDH are listed in Table 1. Primer amplification efficiencies were 95–105% with $R^2 > 0.99$. Melt-curve analysis confirmed a single product for each primer pair. GAPDH was validated as a stable reference gene across treatment groups (mean Ct ± SD: control 22.3 ± 0.4, 1% milk 22.5 ±

0.3, 5% milk 22.4 ± 0.5; P = 0.87). No-template controls were included, and each sample was run in technical triplicate.

The thermal cycling process started with an initial denaturation phase at 95°C for 3 minutes, followed by 40 cycles consisting of denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Post-amplification, a melting-curve analysis was conducted to confirm product specificity. The relative expression of transcripts was determined using the comparative $2^{-\Delta\Delta Ct}$ method, with GAPDH as the reference gene and the untreated (0% milk) samples as the baseline group.

Table 1. Primer sequences used for quantitative real-time PCR analysis of TJP1, OCLN, and GAPDH gene expression.

Gene	Forward (5' → 3')	Reverse (5' → 3')
ZO-1 (TJP1)	GAGTTTGATAGTGGACAGTT	AGTGTCTGGAAATGACCTTC
OCLN	TTCATGTGACGGCTCGTGAT	GAGGCAGCTGTGTGTCCTGT
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

Western Blot Analysis

Cells were treated for 48 hours before being rinsed twice with cold Phosphate-Buffered Saline (PBS) and then lysed using Radioimmunoprecipitation Assay (RIPA) buffer from Sigma-Aldrich (cat. no. R0278), which included a protease inhibitor cocktail (Sigma-Aldrich, cat. no. P8340) and phosphatase inhibitor cocktail (Sigma-Aldrich, cat. no. P5726). The lysates were cleared by centrifugation at 12,000 × g for 15 minutes at 4°C, and the total protein concentration was assessed using the Bradford assay from Bio-Rad (cat. no. 5000006).

Equal amounts of protein (30 µg) from each sample were combined with Laemmli sample buffer (Bio-Rad, cat. no. 1610747), heated at 95°C for 5 minutes, and then separated on 10% SDS-PAGE gels (Bio-Rad, cat. no. 4561033). The proteins were transferred onto Polyvinylidene fluoride (PVDF) membranes (Bio-Rad, cat. no. 1620177) using the Trans Blot® Turbo™ Transfer System from Bio-Rad. The membranes were blocked with 5% non-fat milk (Bio-Rad, cat. no. 1706404) in TBS-T (Tris-buffered saline with 0.1% Tween 20) for 1 hour at room temperature.

Membranes were incubated overnight at 4°C with primary antibodies against ZO-1 (rabbit, 1:1000; Cell Signaling Technology, cat. no. 13663; RRID: [insert correct RRID]; expected molecular weight 220 kDa), OCLN (rabbit, 1:1000; Cell Signaling Technology, cat. no. 91131; RRID: [insert correct RRID]; expected molecular weight 65 kDa), and β-actin (mouse, 1:5000; Cell Signaling Technology, cat. no. 4970; expected molecular weight 45 kDa). Antibody specificity was validated using positive control lysates and by the presence of single bands at the expected molecular weights. Separate membranes were used for each target protein to avoid stripping artifacts.

After three more washes, the immunoreactive bands were identified using enhanced chemiluminescence (ECL; Bio-Rad Clarity™ Western ECL Substrate, cat. no. 1705060) and viewed with a ChemiDoc™ MP Imaging System (Bio-Rad). The intensities of the bands were measured using ImageJ software (version 1.53; National Institutes of Health, Bethesda, MD, USA). ZO-1 and OCLN expression levels were standardized to β-actin and presented as optical density ratios.

Statistical Analysis

Data are presented as mean ± SEM from three independent cell-culture experiments (n = 3 biological replicates per group; each biological replicate was an independent passage and treatment). Within each experiment, qPCR was performed in technical triplicate and Western blots were run once per biological replicate. Group differences were assessed using one-way ANOVA followed by Tukey's multiple-comparisons test. Exact P-values are reported in the text and tables. Because n = 3, normality and homogeneity of variance were not formally tested; however, non-parametric Kruskal-Wallis tests gave consistent results (all $P > 0.05$). Statistical analyses were performed using GraphPad Prism version 9.5.0 (GraphPad Software, San Diego, CA, USA). A two-sided P-value < 0.05 was considered statistically significant.

Results

Breast Milk Treatment Did Not Significantly Alter ZO-1 mRNA Expression

Real-time PCR analysis showed no statistically significant overall difference in ZO-1 mRNA expression among the control, 1% breast milk, and 5% breast milk groups after 48 hours of treatment (one-way ANOVA, P = 0.42; Table 2). Compared with the control group, ZO-1 expression was 1.15

± 0.18-fold in cells treated with 1% breast milk (Tukey-adjusted P = 0.38) and 1.25 ± 0.22-fold in cells treated with 5% breast milk (Tukey-adjusted P = 0.25). Thus, breast milk treatment did not result in a statistically significant change in ZO-1 mRNA expression under the conditions tested.

Breast Milk Treatment Did Not Significantly Alter OCLN mRNA Expression

Similarly, one-way ANOVA revealed no statistically significant overall difference in OCLN mRNA expression among the three experimental groups after 48 hours of treatment (P = 0.51; Table 2). OCLN expression was 1.10 ± 0.15-fold in the 1% breast milk group (Tukey-adjusted P = 0.55) and 1.20 ± 0.18-fold in the 5% breast milk group (Tukey-adjusted P = 0.32) compared with the control group. These differences were not statistically significant.

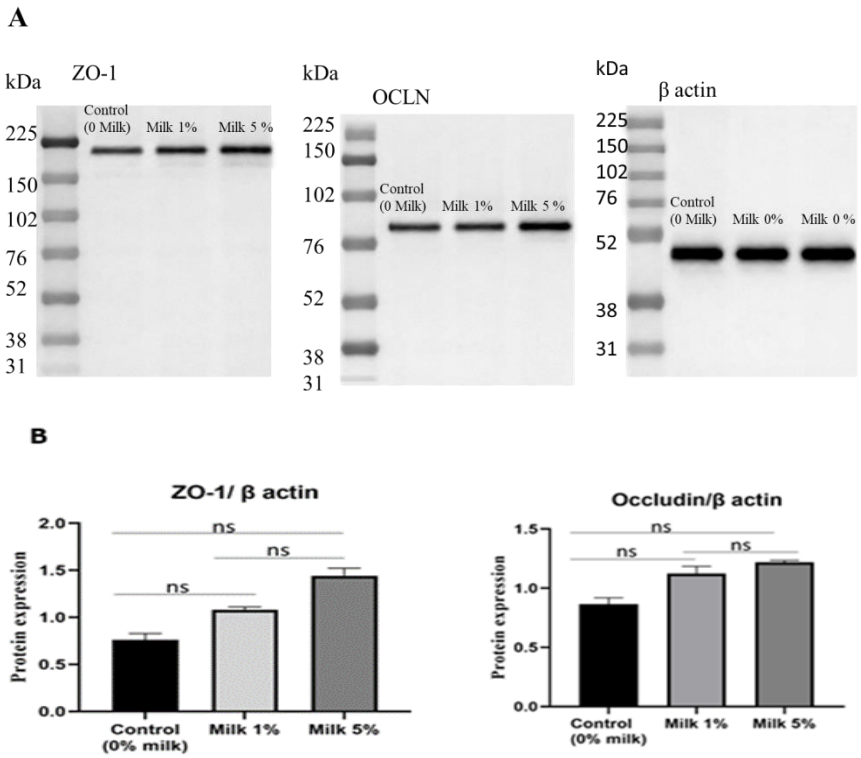


Figure 1. Representative Western blot of ZO-1, Occludin, and β actin proteins and densitometric analysis. (A) Densitometric analysis of ZO-1 and OCLN protein expression. Individual data points from three independent biological replicates are overlaid on the bars; bars represent mean ± SEM (n = 3 per group). (B) Representative Western blot of ZO-1, Occludin, and β-actin proteins. β-actin was used as the loading control. Exact P-values are reported in the figure for each comparison. P > 0.05 was considered not statistically significant.

Table 2. Relative mRNA expression of ZO-1 and OCLN after 48 hours of breast milk treatment

Group	ZO-1/GAPDH (Mean ± SEM)	OCLN/GAPDH (Mean ± SEM)
Control (0% Milk)	1.00 ± 0.05	1.00 ± 0.04
Milk 1%	1.15 ± 0.18	1.10 ± 0.15
Milk 5%	1.25 ± 0.22	1.20 ± 0.18

Data are presented as mean ± SEM from three independent biological replicates (n = 3 per group). Overall group differences were assessed using one-way ANOVA, with post hoc pairwise comparisons performed using Tukey's multiple-comparisons test. Overall ANOVA P-values were 0.42 for ZO-1 and 0.51 for OCLN. Tukey-adjusted P-values for comparisons with the control group are reported in the text. A P-value < 0.05 was considered statistically significant.

Breast Milk Treatment Did Not Significantly Alter ZO-1 or OCLN Protein Expression

Western blot analysis showed no statistically significant overall differences in ZO-1 or OCLN protein expression among the three experimental groups after 48 hours of treatment (Figure 1). For ZO-1, one-way ANOVA showed no significant overall difference among the groups (P = 0.47; Table 3). Compared with the control group, ZO-1 protein expression was 1.10 ± 0.15-fold in the 1% breast

milk group (Tukey-adjusted P = 0.50) and 1.20 ± 0.18-fold in the 5% breast milk group (Tukey-adjusted P = 0.30). Similarly, no significant overall difference was observed in OCLN protein expression (one-way ANOVA, P = 0.55; Table 3). OCLN expression was 1.08 ± 0.14-fold in the 1% breast milk group (Tukey-adjusted P = 0.58) and 1.15 ± 0.16-fold in the 5% breast milk group (Tukey-adjusted P = 0.40) compared with the control group.

Table 3. Relative protein expression of ZO-1 and OCLN after 48 hours of breast milk treatment

Group	ZO-1/β-actin (Mean ± SEM)	OCLN/β-actin (Mean ± SEM)
Control (0% Milk)	1.00 ± 0.06	1.00 ± 0.05
Milk 1%	1.10 ± 0.15	1.08 ± 0.14
Milk 5%	1.20 ± 0.18	1.15 ± 0.16

Data are presented as mean ± SEM from three independent biological replicates (n = 3 per group). Overall group differences were assessed using one-way ANOVA, with post hoc pairwise comparisons performed using Tukey's multiple-comparisons test. Overall ANOVA P-values were 0.47 for ZO-1 and 0.55 for OCLN. Tukey-adjusted P-values for comparisons with the control group are reported in the text. A P-value < 0.05 was considered statistically significant.

Discussion

The mature Caco-2 intestinal epithelial cell model was selected to directly evaluate the effects of human breast milk on intestinal tight-junction proteins, specifically ZO-1 and OCLN, under controlled experimental conditions. Differentiated Caco-2 cells provide a reproducible human epithelial model that forms well-defined cell–cell junctions and expresses these proteins. Although Caco-2 cells do not fully replicate the immature intestinal environment of preterm infants with NEC, they were considered appropriate for this initial mechanistic assessment.

In the present study, exposure to human breast milk at 1% and 5% concentrations for 48 hours did not significantly alter the mRNA or protein expression of ZO-1 or OCLN in Caco-2 cells under basal culture conditions. These findings indicate that, under the experimental conditions examined, human breast milk did not significantly modulate the abundance of these two tight-junction markers. Importantly, because epithelial barrier function was not directly assessed using functional assays such as trans-epithelial electrical resistance (TEER), permeability measurements, or Fluorescein Isothiocyanate (FITC)-dextran flux, the present findings should not be interpreted as demonstrating either an improvement or a lack of effect of breast milk on intestinal epithelial barrier function.

ZO-1 and OCLN play vital roles in regulating paracellular permeability and coordinating tight junction formation in the intestinal epithelial barrier [12, 13]. Disruption of these proteins has been described in Hirschsprung-associated enterocolitis [14]. Whether similar alterations are consistently present in other intestinal inflammatory conditions, including NEC, requires further study.

Previous studies have shown that bioactive components of human milk, including lactoferrin, TGF-β, EGF, HMOs, and secretory IgA, may reduce inflammation and support epithelial repair rather than consistently increasing TJP expression [15-17]. Therefore, the lack of substantial changes observed in the present study may partly reflect the absence of an inflammatory stimulus. Future studies should investigate the effects of human breast milk under inflammatory conditions, such as TNF-α-, IL-1β-, or LPS-induced models [18]. These pathways represent potential mechanisms and were not directly investigated in the present study.

The biological effects of breast milk may involve multiple complementary mechanisms that are not necessarily reflected by changes in ZO-1 or OCLN abundance [19, 20]. Human milk contains bioactive components that can modulate epithelial homeostasis through anti-inflammatory and antimicrobial activities and by influencing the intestinal microbiota [19-21]. These mechanisms may affect barrier function independently of changes in total ZO-1 or OCLN expression.

Prior research has shown that specific elements in breast milk, like lactoferrin and oligosaccharides, can safeguard intestinal barrier function. However, various biological and methodological factors could explain the varying or inconclusive results observed in some clinical studies [16, 21, 22]. The concentrations of breast milk tested (1% and 5%) might not have been strong enough to trigger detectable reactions, and the 48-hour treatment duration might have missed temporary alterations in gene activity. Additionally, Caco-2 cells, despite being commonly utilized, do not completely mimic the developing intestinal lining of premature babies. Differences in breast milk content and the use of low-serum culture settings could have also impacted the cellular responses. Furthermore, the centrifugation and filtration steps used during milk preparation may have reduced or removed some bioactive components, including lipid-associated factors and extracellular vesicles

such as exosomes, which could influence intestinal epithelial responses. Thus, the absence of significant changes in ZO-1 and OCLN expression may partly reflect the processing of the milk rather than the effects of whole, unprocessed pooled human milk.

In general, our results show that pooled human milk does not have a substantial impact on the regulation of ZO-1 or OCLN expression in Caco-2 cells in normal circumstances. This suggests that protective effects of breast milk on the gut barrier may work through various mechanisms that are more noticeable when the epithelium is damaged or inflamed. This underscores the significance of relevant inflammatory models in upcoming research studies.

Strengths, Limitations, and Future Perspectives

A major strength of this study was the combined evaluation of ZO-1 and OCLN at both mRNA and protein levels. However, breast milk from three donors was pooled, preventing assessment of donor-to-donor variability and individual donors as independent biological replicates. Other limitations include the use of only two milk concentrations and one exposure duration, absence of functional barrier assays and inflammatory models, and evaluation of only two junctional proteins.

Future studies should assess individual donor samples and incorporate inflammatory models, functional barrier assays, and broader molecular analyses to better define the protective effects of pooled human milk on the developing intestinal epithelium.

Conclusion

In conclusion, pooled human milk did not significantly alter ZO-1 or OCLN expression in Caco-2 cells under basal culture conditions. These findings indicate that, under the experimental conditions examined, the protective effects of breast milk against NEC cannot be attributed to direct induction of ZO-1 or OCLN expression alone.

Abbreviations	Full Form
Caco-2	Human colorectal adenocarcinoma cell line
GI	Gastrointestinal
NEC	Necrotizing Enterocolitis
OCLN	Occludin
PCR	Polymerase Chain Reaction
PTI	Preterm Infant
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
TJP1	Tight Junction Protein 1
ZO-1	Zonula Occludens-1

Declaration

Conflict of Interest:
The authors state that they have no conflicts of interest.

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Availability of data and materials:
All data created or examined in this study are provided within this published article.

Consent for publication:
Not applicable.

Ethics approval and consent to participate:
The study protocol was reviewed and approved by the Ethics Committee of Asadabad School of Medical Sciences, Asadabad, Hamadan, Iran (Ethics Approval No.: ASAUMS.REC.1401.002). The experiments were performed using a commercially purchased human intestinal epithelial cell line and commercially purchased human breast milk. No human participants or animals were directly involved in this study, and all experimental procedures were conducted in accordance with institutional guidelines.

AI Disclosure:
AI-assisted tools were used for language polishing and improving the clarity and readability of the manuscript. The authors reviewed and revised the text and take full responsibility for the scientific accuracy, interpretation of the findings, and final content of the manuscript.

Author Contributions:
R.I. conceived and designed the study, performed the experiments, analyzed and interpreted the data, prepared the figures, and drafted the manuscript. J.I. contributed to the study design, data interpretation, and critical revision of the manuscript. F.T. supervised the research, reviewed the manuscript for important intellectual content, and approved the final version. All authors read and approved the final manuscript.

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