Review Article

A literature review on lactopontin and its roles in early life

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Objective: Our study aims to review the functions and possible mechanisms of lactopontin (LPN) in early life.

Background: Human milk proteins provide a variety of protection and health benefits in early life. One of these multifunctional proteins is LPN, which is osteopontin (OPN) derived from milk.

Methods: Information used to write this paper was collected from Uniprot, PubMed, and Google Scholar, including *in vitro*, *in vivo*, and clinical studies.

Conclusions: LPN is a highly phosphorylated, *O*-glycosylated acidic protein and a unique type of OPN, as it presents at the highest concentration and a higher degree of posttranslational modifications (PTMs) in human milk than other tissues and excretions. LPN is present in milk and the intestinal tracts of infants after consumption as a mixture of intact protein and peptides, which can bind diverse integrin and receptors in the target cell and drive downstream signaling pathways. LPN is found to play important roles in developing the immune, intestinal and nervous systems in early life. Moreover, LPN has also shown to support preterm infants' health when they are especially vulnerable after delivery via animal studies. Additionally, LPN can form protein complex with another milk bioactive protein, lactoferrin (LF), to withstand proteolysis and perform more efficient biological activity. Therefore, LPN showed great potential for early life while more clinical trials and evidence are still emergying.

Keywords: Lactopontin (LPN); milk osteopontin; immune regulation; gut development; neurodevelopment

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Introduction

The mammary glands of female mammals secrete milk to nourish their offspring during early life after birth. Milk is a natural source of nutrients and bioactive proteins necessary for the health and growth of infants. Many studies have identified the short and long-term benefits for both maternal health and infant development (1). Proteins in milk are increasingly appreciated for their bioactive functions rather than a source of essential amino acids. In recent years, osteopontin (OPN) in milk has attracted much attention. OPN was initially found in bone tissue and is

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HUMAN  IPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQKQNLLAPQNAVSSEETNDFKQETLPSKSNESHDHMDDMDDEDDDDHVD    82
BOVIN  LPVKPTSSGSSEEKQLNNKYPDAVATWLKPDPSQKQTFLAPQNSVSSEETDDNKQNTLPSKSNESPEQTDDLDDDDDN----    78

HUMAN  SQDSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYDGRGDSVVYGLRSKSKKFRRPDIQ   164
BOVIN  SQD-VNSNDSDDAETTDDPDHSDESHHSDESDEV--DFPTDIPTIAVFTPFIPTESANDGRGDSVAYGLKSRSKKFRRSNVQ   157

HUMAN  YPDATDEDITSHMESEELNGAYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKANDESNEHSDVID   246
BOVIN  SPDATEEDFTSHIESEEMHDAPK----------------------KTSQLTDHSKETNSSELSKELTPKAKDK-NKHSNLIE   216

HUMAN  SQELSKVSREFHSHEFHSHEDMLVVDPKSKEEDKHLKFRISHELDSASSEVN                                  298
BOVIN  SQENSKLSQEFHSL-----EDKLDLDHKS-EEDKHLKIRISHELDSASSEVN                                  262
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Figure 1 Alignment of human and bovine LPN sequences. Broken lines indicate introduced gaps. Residues for phosphorylation and glycosylation are highlighted in red and orange, respectively. RGD (Arg-Gly-Asp) motif is colored in green, and the cryptic sequence is underlined. LPN, lactopontin.

considered an important bone matrix protein (2,3).

Further studies revealed that OPN could be synthesized or secreted by various tissues and cells in the body. OPN is present in two forms: intracellular OPN found in immune cells (4) and secreted OPN present in body fluid, such as milk, urine, blood, saliva, and bile (5). OPN in milk, also known as lactopontin (LPN) (6,7), is a remarkable type of OPN as it is found in higher concentrations in human milk than OPN in other tissues and excretions (8), it has a higher degree of posttranslational modifications (PTMs) (9-11), and the mother secretes it but ingested by the offspring as food and plays important roles in the infant's growth and health (3).

Microarray analyses of cells in human milk revealed that the secreted phosphoprotein 1 (*SPP1*) gene which code LPN is highly expressed throughout the lactational stages (12). The average concentration of LPN in human milk (138 mg/L) is about 8 times higher than in bovine milk (18 mg/L) (3). One study recruited 629 lactating mothers (mean age 31.4 years) from four countries—China, Japan, Korea, and Denmark—and collected 829 breast milk samples (13). The mean LPN in the breast milk of Chinese mothers 4.3 weeks postpartum (266.2 mg/L) was significantly higher than that of Korean mothers at 3.9 weeks postpartum (216.2 mg/L), Japanese mothers 9.1 weeks postpartum (185 mg/L), and Danish mothers 17.4 weeks postpartum (99.7 mg/L). Moreover, the proportion of LPN in total milk proteins was also higher in Chinese mothers (2.7%) than in Korean mothers (1.8%), Japanese mothers (2.4%), and Danish mothers (1.3%) (13). Another study examined the changes in LPN in the milk of 12 mothers of full-term infants during the 12 months postpartum and found that LPN concentration was highest in colostrum (178.0±17.9 mg/L), gradually decreased from colostrum to transitional milk to mature milk, and remained a stable concentration in late lactation (14). The higher concentration of LPN in colostrum suggests LPN may play a critical role in the early life of infants. Recent studies have reported different effects of LPN in early life by *in vitro*, animal studies, and clinical trials in infants. This study aims to summarize the recent findings, conduct a literature review on the functions and possible mechanisms of LPN in early life, and discuss future perspectives. We present the following article in accordance with the Narrative Review reporting checklist (available at https://dx.doi.org/10.21037/tp-21-293).

Methods

The literature that was reviewed in this paper was sourced from Uniprot, PubMed, and Google Scholar. The protein sequence and modified sites were searched in Uniprot by the gene name "*SPP1*" using the Swiss-Prot database. The keywords for the literature search in Pubmed and Google Scholar were "osteopontin," "lactopontin," and "milk." All articles collected for this study were published before April 30, 2021. The reference lists of relevant articles were manually searched for additional literature. Only publications in English were reviewed.

Structural characteristics of LPN

LPN is a highly phosphorylated, *O*-glycosylated acidic protein that is structurally similar among different species. For example, as shown in *Figure 1*, human LPN contains 298 amino acids (AAs), and bovine LPN contains 262 AAs, of which 182 (61%) are identical to human LPN and 44 are highly structurally similar (8). LPN in all species

has an integrin-binding Arg-Gly-Asp (RGD) motif and a cryptic sequence (human: SVVYGLR, bovine: SVAYGLK). Moreover, LPN is heavily modified by PTMs and heterogeneous proteolytic processing by milk enzymes.

Most of the modified residues and regulatory proteolytic cleavage sites are conserved between human and bovine LPN. Human LPN contains 36 phosphorylation sites (34 phosphoserine sites and 2 phosphothreonine sites) and 5 *O*-glycosylation sites (15). In comparison, bovine LPN has 28 phosphorylation sites (27 phosphoserine sites and 1 phosphothreonine site) and 3 *O*-glycosylation sites, wherein 25 phosphorylation sites and 3 *O*-glycosylation sites are identical to human LPN (2,14). The phosphorylation sites are arranged in clusters of three to five phosphoresidues predominantly located in the target sequence of the kinase FAM20C (16), while the RGD motif and the glycosites are not phosphorylated (10). In human LPN, approximately 25–26 phosphates are distributed over 36 potential sites (10,11), while in bovine LPN, approximately 22 phosphates are distributed over 28 potential sites (17). Most glycan structures identified in human LPN have been shown to consist of the GalNAc-galactose-(GlcNAc) core-2 structure extended by fucosylated and, to a lesser degree, sialylated poly-N-acetyllactosamine units (11). The multifunctional bioactivity of LPN is associated with its binding to several integrins and CD44 receptors, which were affected by its phosphorylation and *O*-glycosylation statuses (11,18).

In addition, LPN is susceptible to degradation by a variety of proteases naturally present in breast milk, such as thrombin, cathepsin D, and plasmin, producing endogenous LPN polypeptides of various lengths and sequences (19,20). After entering the gastrointestinal tract of infants, LPN is further cleaved, and many peptides are produced. A study identified human milk and bovine milk peptides in gastric aspirates and showed the major cleavage sites of LPN were mostly conserved between the two species regarding hydrophobicity patterns. In bovine LPN, Phe^{38}-Leu^{39} was the largest cleavage site which was also present in human LPN as Leu^{38}-Leu^{39}, whereas in human LPN, the largest cleavage site was Thr^{26}-Trp^{27}, which was also found in bovine LPN (20). The various peptides derived from LPN, in turn, bind to a range of cell surface integral proteins or CD44 receptors to perform a variety of physiological functions (21).

Biological functions of LPN

The levels of OPN in the cord blood and plasma of 3-month-old infants are 7–10 times higher than in adult plasma (3). The high levels of LPN in milk, especially in the colostrum, suggest that LPN may play an important role in early life when the infants are immunologically immature (12). LPN can bind and form a soluble complex with calcium as an acidic protein, thereby increasing the stability of calcium and inhibiting unintentional precipitation of amorphous calcium phosphate in milk (22). Moreover, LPN shows multifunctional bioactivities due to its integrin and receptor binding properties and initiates signaling pathways in the form of intact LPN or LPN peptides (23-25), as it is partly resistant to proteolysis and is prone to proteolytic cleavage in milk or the intestinal tract (15). A study found that the polypeptides formed by LPN usually contain RGD sequences and SVVYGLR motifs and still can bind integral proteins such as αvβ3 (21) or even increase the binding abilities (8). Due to the structural similarity and conserved functional sites of LPN, the biological activity of LPN is similar across mammalian sources.

LPN has been shown to play a role in immunological development in infants, including defense of pathogen-introduced infections and regulation of immune response. An *in vitro* study found that the C-terminal fragment obtained by thrombin-treated bovine LPN could bind to αXβ2 (CD11c/CD18) integrins that were highly expressed on the surface of monocytes, mediate the chemoattraction of interleukin (IL)-1-activated human monocytes and exert a regulatory effect on macrophage chemotaxis, adhesion, infiltration, and phagocytosis (26). Moreover, LPN can bind Staphylococcus aureus in a dose-dependent manner under a Ca^{2+}-dependent interaction (26). Another *in vitro* assay found that LPN promoted the migration and activation of dendritic cells (DCs), induced T cell proliferation, and promoted their progression to the Th1 phenotype by stimulating DC release (27). Mice deficient in *SPP1* gene expression had an imbalanced Th1/Th2 immune response and were more susceptible to viral and bacterial infections than wild type mice (28). LPN may act at the gut mucosal surface of infants and regulate the immune response by increasing the expression of the Th1 cytokine interleukin-12 (IL-12) and interferon-gamma via integrin receptor binding, and decreasing the Th2 cytokine IL-10 via CD44 receptor binding (28). In a randomized controlled trial, 240 healthy infants were divided into four groups: standard formula group (no supplemental LPN, group F0), formula group with two doses of supplemental LPN (group F65: 65 mg/L, group F130: 130 mg/L), and exclusive breastfeeding group (BF group) from 1–6 months of age

(29,30). The study showed that LPN supplementation changed the plasma levels of several amino acids and cytokines in formula-fed infants to levels similar to those in breastfed infants, although some changes were not significant. Infants fed with standard formula had significantly higher serum levels of the tumor necrosis factor-alpha (TNF-α) than infants fed formula with additional LPN, who did not differ from the breastfed group (29). The incidence and frequency of fever reported by parents were significantly higher in the F0 group than in the BF group, while there were no differences between the F65, F130, and BF groups (29). In addition, the peripheral blood T-cell ratio was significantly higher in the F130 group than in the F65 group and the F0 group and was not significantly different from that of the exclusively breastfed BF group (30). Thus, infants fed formula supplemented with bovine LPN showed a higher similarity to the breastfed infants than infants fed regular formula alone, which may support improved innate and adaptive immune development (29,30).

In animal studies, LPN promoted small intestinal mucosal growth, improved duodenal villus thickness and crypt depth, increased small intestinal mucosal surface area, and enhanced its nutrient absorption efficiency (31). LPN has been reported to regulate small intestinal gene expression through the integrin signaling pathway and influence early-life intestinal proliferation and maturation by regulating cell migration and cell chemotaxis (31). LPN has also been found to play a role in protecting small intestinal mucosal integrity by affecting integral tight junction membrane proteins such as occludin and zonula occludens-1 in the intestinal mucosa (32). In an *in vitro* study, bovine LPN upregulated the secretion of IL-18 from intestinal epithelial cells (Caco-2), promoted Caco-2 differentiation, and stimulated intestinal immune function (33). In another *in vitro* study, human LPN and bovine LPN stimulated proliferation of human intestinal cells, and gene expression were changed for those tightly related to proliferation and immune function, such as MAPK13, CCNE1, CdGAP, CXCL10, IL6ST, and NFKB (34). In a study with newborn rhesus monkeys, feeding bovine LPN (125 mg/L) affected intestinal gene expression (35). From birth to 3 months, rhesus monkeys were fed either breast milk or normal formula with or without bovine LPN. Although no difference was observed in growth, body composition, or blood immune cells, small intestinal transcriptome revealed that while 1,017 genes were differentially expressed between the normal formula and breast milk groups, the supplementation of LPN in formula diminished the number of different genes from 1,017 to 217 (35). Bovine LPN added in drinking water (20 μg/mL) to dextran sulfate sodium (DSS)-treated wild-type (WT) mice were correlated with less weight loss, spleen enlargement, gut neutrophil activity, and pro-inflammatory mediators, and increased colon length and red blood cell counts compared with DSS-treated WT mice (36). In addition, LPN may alter intestinal flora and affect the content of short-chain fatty acids in piglets (37).

OPN is highly expressed in the brain in early infancy (38) and is crucial in forming myelin sheaths for normal brain development (39). Human LPN, administered orally in mouse pups, could enter the brain and increase the brain OPN protein level (40). The mice breastfed by mothers lacking LPN after knockout had reduced expression of myelin-related proteins and reduced proliferation and differentiation of NG-2 glial cells to oligodendrocytes compared with mice breastfed by wild type mothers, accompanied by reduced ERK-1/2 and P13K/Akt signaling, and memory and learning ability were found to be impaired using passive avoidance tests and rotarod experiments (40). Another animal study tested the bovine LPN on the brain and cognitive development in piglets (37). Piglets were fed a soy protein-based formula with or without bovine LPN (250 mL/L) from birth to 34 days. A novel object recognition (NOR) test was applied to assess the cognitive development and found that the two groups of piglets did not show significant differences in behavioral tests, but the piglets in the LPN-enriched group had a shorter waiting time than those in the non-LPN-enriched group when first exploring new targets. Neuroimaging outcomes revealed the LPN-enriched group had increased relative brain volumes of the corpus callosum, lateral ventricle, left and right internal capsule, left and right putamen-globus pallidus, right hippocampus, and right cortex. Diffusion tensor imaging revealed higher radial diffusivity in the corpus callosum and lower fractional anisotropy in the LPN-enriched group. The results suggest that bovine LPN promotes the development of neural structures and improves exploratory behavior (37).

Moreover, LPN for preterm infants is gaining attention. Newborn premature infants have shorter intrauterine development, poorer growth and development, immature development of vital organs such as brain, lung, and intestine, and greater susceptibility to various infectious diseases (41). The shorter the gestational age is, the worse

the developmental status of preterm infants (42). LPN is an important component of preterm mothers' milk in the form of intact protein (43) and endogenous peptides (44). Animal studies have shown that compared with formula without LPN, the formula with supplementary LPN can reduce the rate of diarrhea in preterm pigs (45) and, while it does not affect the incidence of necrotizing enterocolitis, it can reduce the severity (46). The role of LPN in enhancing the maturation of the immune, intestinal and neurological systems in early life may be critical for preterm infants; thus future clinical studies are needed.

Lactoferrin (LF)-LPN complex

LPN can form complexes with several milk proteins; for instance, it can electrostatically bind to lactoferrin ($K_D=10^{-6}$ M) or lactoperoxidase or bind immunoglobulin M (IgM) with high affinity ($K_D=1.77\times10^{-7}$ M) (47). In these complexes, LPN may protect these immunomodulating and antimicrobial proteins from proteolysis (33) and perform more efficient biological activity than when alone (24,48). The LF-LPN complex is one of the most common complexes naturally occurring in both human and bovine milk (24).

LF is an abundant and important multifunctional, non-heme iron-binding glycoprotein in breast milk (49). LF belongs to the transferrin family and has a high iron affinity ($K_D=10^{-22}$ M) which is 260 times higher than serotransferrin (TF) (50). LF kills pathogenic microorganisms by competitively seizing iron to lack sufficient iron replenishment to maintain biofilm structure and consequently die (51). LF binds to lipopolysaccharide (LPS) on the cell wall of gram-negative bacteria, killing the bacteria by altering cell membrane permeability (52,53). LF also has antiviral activity, preventing viruses from entering the host cell by blocking cellular receptors or directly binding to the virion (54). In addition, LF can recognize LF receptors on the surface of immune cells and affect immunity by promoting immune cell maturation, stimulating immune response, and immunomodulation (55). An *in vitro* experiment on Caco-2 revealed that LF had different functions according to its concentrations. High concentrations of LF (1–1,000 μg/mL) stimulated intestinal cell proliferation, while lower concentrations of LF (1–1,000 μg/mL) enhanced intestinal cell differentiation. LF was observed in higher concentrations during early lactation and lower concentrations during late lactation, which matched the intestinal development of the infants (56).

Since LPN and LF have opposite charges, multiple positively charged LFs can form LF-LPN complexes with one negatively charged LPN. Complexes with an LF to LPN molar ratio of 3:1 were the most active (24). The human LF-LPN complex behaved more stably than LF and LPN monomers *in vitro* digestion assays, and the complex was also more readily taken up by small intestinal cells than monomers, with the bovine LF-LPN complex showing similar properties (24). Another study showed that an LF-LPN complex formed by iron-free LF (apo-LF) and calcium-saturated LPN (holo-LPN) had the strongest proliferative effect on intestinal epithelial cells compared to an LF-LPN complex formed by other ion-binding types, which can withstand the action of digestive enzymes to reach the intestine and exert intestinal proliferation by activating the PI3K/Akt signaling pathway (48).

Limitations to the research reviewed

LPN is highly concentrated in human milk and is important for early infancy. However, most of the research reviewed in this article concerning supplemented LPN was *in vitro* or *in vivo*, and only one clinical trial in Chinese infants was reported. More clinical trials are needed, and systematic reviews should be done when data are efficient.

Summary

LPN is a highly concentrated and important functional component of breast milk, which plays multiple essential roles in early infancy. *In vitro*, *in vivo*, and clinical studies have revealed that LPN contributes to immune development, intestinal proliferation and maturation, and neurodevelopment. Since preterm infants are particularly immature due to shorter intrauterine development, LPN might be critical for preterm infants. As LPN naturally exists in human milk but has lower amount in bovine milk, therefore the breastfeeding infants can get enough LPN from human milk while infants feeding by infant formula may need additional supplemented LPN. However, more clinical studies are still needed to reveal the potential of LPN fully.

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