Potential role of β-carotene-modulated autophagy in puerperal breast inflammation (Review)

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Abstract. Puerperal breast inflammation is common in the first 6-8 weeks postpartum, and without proper management, may lead to a decrease in breastmilk production and early cessation of breastfeeding. Recent studies showed that carotenoids decrease the severity of puerperal breast inflammation. This article summarizes the significant findings on β-carotene with a potential role as an autophagy modulator in puerperal breast inflammation. Puerperal milk stasis causes an increase in inflammatory cytokines and inflammatory cells, leading to reactive oxygen species (ROS) activation that causes oxidative damage to mammary glands and affects breast milk secretion. β-carotene has an anti-inflammatory effect related to its ROS-scavenging activity and modulates autophagy, thus stimulating the removal of damaged cellular structures and supporting milk gland survival. β-carotene modulates autophagy through phosphorylation of NF-κB, JNK, p38, Akt, and Nrf2, affects the ratio of Microtubule-associated protein 1A/1B-light chain 3 (LC3)-II/LC3-I, and has a role in the regulation of the JAK2/STAT3, PI2K/Akt/mTOR and AMPK pathways. Although the *in vitro* and *in vivo* studies showed promising results, further studies on humans are required to better conclude the potential role of β-carotene in managing puerperal breast inflammation.

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Abbreviations: AGE, Advanced Glycation End Product; AIF, apoptosis-inducing factor; ATG, autophagy genes; BNIP3, Bcl2/adenovirus E1B 19 kDa protein-interacting protein; CEN, carotene extract nanoemulsion; CL, Cardiolipin; Cyt-c, Cytochrome-c; LC3, Microtubule-associated protein 1A/1B-light chain 3; MOMP, mitochondrial outer membrane permeability; RAE, retinol activity equivalent; ROS, Reactive Oxygen Species; SF, silk fibroin; TRAIL-R, TNF-related apoptosis-inducing ligand receptor

Key words: autophagy, breast milk, inflammation, β-carotene, ROS, puerperal

Contents

1. Introduction

In exclusive breastfeeding (EBF), breast milk becomes the only source of nutrition for infants without any other liquids or solids (1). EBF is beneficial for the baby as well as the mother (2-4), and the World Health Organization (WHO) and United Nations Children's Fund (UNICEF) recommend EBF for the first 6 months of an infant's life and should then be continued for 2 years or more (5). However, in 2020, only 44% of babies under 6 months worldwide received exclusive breastfeeding (2). Many factors influence the cessation of breastfeeding, including insufficient milk supply (6-8), self-weaning (9), primiparity (10), mother/infant separation (6), inconvenience/fatigue due to breastfeeding (11), and inflammatory breast diseases such as mastitis (12,13).

Mastitis is inflammation of the breast, with or without infection (14). Worldwide, ~20% of women suffer from mastitis whilst breastfeeding (15). The majority of cases occur in the first 6-8 weeks postpartum, and approximately one-third of women experience a recurrent episode (16,17). Up to 3% of cases of mastitis develop into a breast abscess (18). Puerperal breast inflammation starts with insufficient breast emptying (milk stasis) (12). This causes an increase in intraductal pressure and opens milk duct epithelial cells' intercellular junctions. Breast milk further moves into the connective tissue and generates a sterile inflammatory environment, from which a secondary bacterial infection may follow. Without proper treatment, this can progress to a mammary abscess that requires surgical treatment (12). Several studies indicate that

puerperal breast inflammation is correlated with the cessation of breastfeeding (12,16).

Milk stasis increases the levels of inflammatory cytokines (TNF-α, IL-1b, IL-6, IL-8), neutrophils, macrophages, lymphocytes, eosinophils, and various epithelial cells of mammary tissue (19). Cytokines further activate reactive oxygen species (ROS), which play a complex role in the inflammatory process (20). The excessive buildup of ROS disrupts cellular homeostasis, causes oxidative stress and mitochondrial dysfunction, and induces autophagy (21). The oxidative damage in breast inflammation can cause mammary gland cell death and affects breast milk secretions (15).

Antioxidant supplementation decreases the incidence, duration, and severity of mastitis (22,23). Antioxidants scavenge free radicals and inhibit the activity of oxidizing enzymes, thus reducing the damage caused by free radicals (23). Some herbal treatments with high antioxidant and anti-inflammatory activities are widely used in mastitis treatment, such as Moringa leaves (24), binahong leaves (*Anredera cordifolia*) (25), cherry leaf decoction (*Muntingia calabura L.*) (26), Red Algae Eucheuma Spinosum (27), Macroalgae Extract (28), garlic, manjakani fruit, betel leaf, white turmeric, and eucalyptus leaves (29). Carotenoids are another type of active substance found in plants with high antioxidant contents and anti-inflammatory effects, but have not been as widely explored as treatment or prevention options for mastitis. Previous studies showed that increasing serum retinol concentrations in dairy cows was associated with a decreased risk of clinical mastitis, and low concentrations of vitamin A and β-carotene increased the severity of mastitis (30,31). Carotenoids have a significant impact on maintaining the health of epithelial tissue and the stability of mucosal surface integrity (32).

Carotenoids are yellow-orange pigments that are abundantly present in several plants, marine invertebrates, and microorganisms. There are ~50 carotenoids consumed in a standard human diet, but only 20 have been identified in the human plasma, with β-carotene, α-carotene, lycopene, and cryptoxanthin as the most common (33). Carotenoids have potent antioxidant, anti-inflammatory, and immunomodulatory abilities. (34) β-carotene is the most common carotenoid, which is also known as pro-vitamin A, and is one of the most abundant carotenoids in the human blood with several health-promoting properties (35,36). The anti-inflammatory effects of β-carotene have been demonstrated in multiple systems (37,38).

Previous studies showed that β-carotene and vitamin A reduce the risk of puerperal breast inflammation (39). Low concentrations of plasma vitamin A (<80 μg/100 ml) and β-carotene (<200 μg/100 ml) correlated with the severity of mastitis (23,30). Animal studies in cows showed that mastitic cows, as determined using the California Mastitis Test score, had significantly lower plasma vitamin A and β-carotene concentrations than healthy cows (32). To reduce the risk of mastitis, a dry and lactating cow's diet should contain 110 IU/kg bwt/day of vitamin A (22). Plasma concentrations of β-carotene >3 mg/l promoted udder health in dairy cattle (40). Low concentrations of plasma vitamin A (<0.8 μg/ml) and β-carotene (<2 μg/ml) were linked with the severity of mastitis. However, unfortunately, there is minimal data regarding the optimal dose of β-carotene for postpartum women in correlation to treatment or prevention of puerperal breast inflammation.

As an antioxidant, β-carotene suppresses intracellular ROS production and reduces antioxidative enzyme activity (36,41,42). B-carotene supplementation exerts a stabilizing effect on polymorphonuclear cells such as neutrophils, eosinophils, and basophils, and optimizes lymphocyte function (23). The inflammatory response in mastitis and the role of β-carotene as an antioxidant is summarized in Fig. 1. However, there is limited study on the role of β-carotene in correlation with autophagy modulation and puerperal breast inflammation. The present review summarizes the major findings on β-carotene as a potential autophagy modulator in puerperal breast inflammation.

2. Autophagy in puerperal breast inflammation

Breast fullness or breast engorgement is a part of lactogenesis in the postpartum period. This fullness manifests ~36 h postpartum and occurs 3-7 days postpartum, with primiparous mothers most commonly affected (43). In breast engorgement, the breast undergoes increased vascularity, congestion, milk accumulation, and oedema (43,44).

In milk stasis, the accumulation of breast milk disrupts vascularisation and causes tissue hypoxia (19). Tissue hypoxia induces the transcription of Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and induction of NIX following HIF1 activation (21). This in turn competes with beclin-1 for the binding of Bcl-2, and the release of beclin-1 is triggered, followed by autophagy (45). PERK, an ER stress sensor, is stimulated and, later in the process, stimulates the expression of Microtubule-associated protein 1A/1B-light chain 3 (LC3) and autophagy gene (ATG)5, both of which are ATGs (46). Oxidative stress triggers FOXO3 and then stimulates the transcription of LC3, BNIP, and NRF2, which induces transcription of p62 (21). The process mentioned above positively modulates autophagy. p53 has a role in activating DNA damage-regulated autophagy modulator and sestrins, two autophagy-related genes that positively regulate autophagy (47). TP53-Induced Glycolysis and Apoptosis Regulator negatively regulates autophagy, but while it is a p53 target, it possesses a p53-independent function in autophagy (21). AMPK is also p53-independent, and is activated by sestrins to inhibit mTOR activity and thus induce autophagy (48). ROS constrains ATG4 protease activity and promotes autophagosome formation (21,49).

Milk stasis also results in high oxygen and energy demands and increases ROS production as a result (12). ROS can assist the healing and tissue repair process in moderate concentrations, but in excess, ROS disrupts redox homeostasis and this results in oxidative stress and damage to cell organelles (50). The increase in free radical levels in puerperal breast inflammation is triggered by the increase of neutrophils, macrophages, lymphocytes, eosinophils, and mammary tissue epithelial cells, TNF-α, IL-1b, IL-6, IL-8, and nitric oxide (NO) (51). ROS accumulation triggers endothelial dysfunction and tissue injury in mammary gland tissue, opens inter-endothelial junctions, and stimulates inflammatory cell migration across the endothelial barrier. These cells support the clearance of pathogens, but excessive amounts may also cause tissue injury (52).

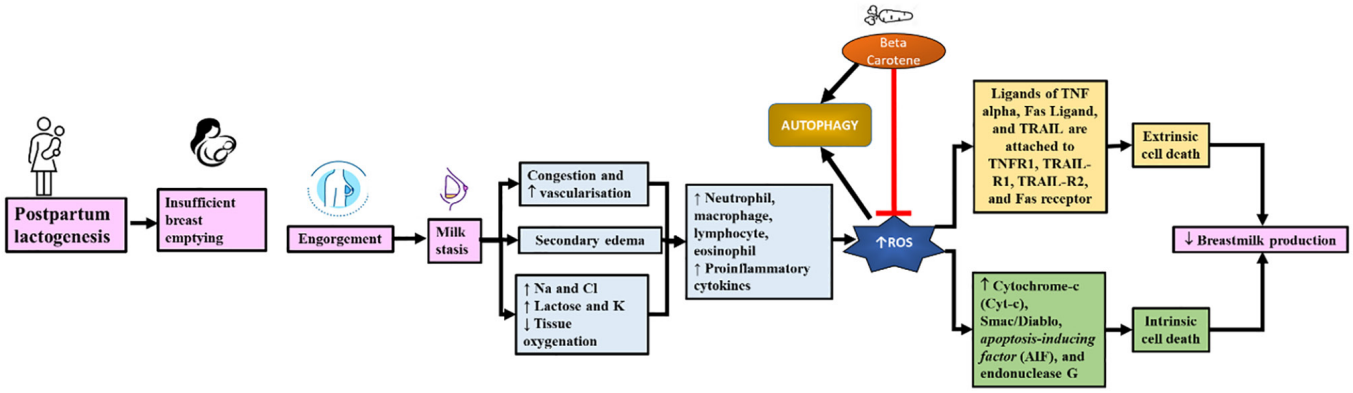

Figure 1. Model of the inflammatory response within a lactating breast and the role of β-carotene as an antioxidant and autophagy modulator.

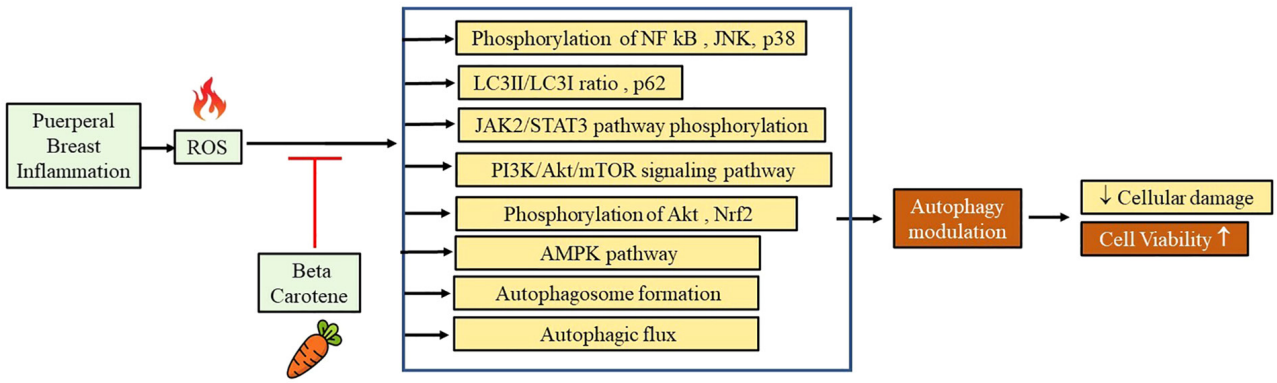

Figure 2. β-carotene scavenges excessive ROS and modulates autophagy through the phosphorylation of several proteins involved in autophagy, affecting JAK/STAT3, PI2K/Akt/mTOR, and the AMPK pathways, β-carotene also affects autophagosome formation and autophagic flux. ROS, reactive oxygen species.

ROS is essential in promoting cell autophagy as a critical signalling molecule (53). Autophagy facilitates the degradation of intracellular proteins and organelles via lysosomes. ROS induces autophagy, and autophagy reduces oxidative damage (49). During inflammation, the NRF2 transcription factor enhances p62 expression, and p62, in turn, creates a positive feedback loop for NRF2 (49).

Autophagy is involved in inflammatory reactions and barrier repair by degrading cell components via the lysosomal pathway (54). Autophagosomes are formed during autophagy to encapsulate protein aggregates and/or damaged organelles. Hydrolytic enzymes degrade the damaged organelles after autophagosome fusion with a lysosome (55). Autophagy is stimulated by any stressor that causes cellular stress, including hypoxia (56), nutrient deficiency (57), chemical exposure (56), excessive ROS (58), or intracellular pathogens (59). Autophagy is a cellular recycling process that involves potential cell death outcomes and functions to protect cells from apoptosis (60).

Four sub-groups of ATGs regulate autophagy; namely Atg1/unc-51-like kinase (ULK) complex, two ubiquitin-like proteins [Atg12 and Atg8/microtubule-associated protein light chain 3 (LC3)] conjugation systems, the class III phosphatidylinositol 3-kinase (PI3K)/vacuolar protein sorting 34 (Vps34) complex I, and two transmembrane proteins, Atg9/mAtg9 and VMP1 (61). ROS induces autophagy through two methods, directly and indirectly. The direct regulation is established through modulation of the involved proteins, such as Atg4, Atg5, and Beclin. Indirect regulation is achieved through autophagy-related signaling pathways, such as the mTOR, mitogen-activated protein kinase (MAPK), p38, ERK, and JNK (62). ROS inhibits mTOR activity and activates AMPK, and initiates autophagy through the increase of Vps34 complex activity (50). Activation of the p53 pathway causes JNK and Sestrin2 activation. JNK and Sestrin2 bind to TSC1/TSC2 and induce autophagy (62). ROS also promotes LC3-II translocation through the inhibition of ATG4, which converts LC3-II into LC3-I (63).

Excessive ROS production triggers cell death extrinsically and intrinsically (52). The extrinsic pathway is mediated via four major cell death receptors; TNF receptor 1, TNF-related apoptosis-inducing ligand receptor (TRAIL-R)1, TRAIL-R2, and the Fas receptor (19). TNFα, Fas ligand, and TRAIL bind to these receptors. TNFα is secreted by activated macrophages and is involved in the increase in inflammatory cytokines (50).

The increase in mitochondrial outer membrane permeability (MOMP) triggers intrinsic pathways of cell death (52). MOMP induces the release of Cytochrome-c (Cyt-c), Smac/Diablo, apoptosis-inducing factor (AIF), and endonuclease G, which play a role in cell death in either a caspase-dependent or -independent manner (64). This increase in MOMP is stimulated by an excessive entry of calcium and a high level of oxidative stress, which causes mitochondrial membrane depolarization (52). An apoptosome is formed as Cyt-c attaches to apoptosis activation factor-1 and this recruits

the initiator pro-caspase 9. Caspase-9 functions to trigger caspase-3. (58) Smac/Diablo also plays a role in activating effector caspases by eliminating the blockage of the inhibitor of apoptosis proteins (50). After AIF is activated, DNA condensation and cleavage induction occur (52). The primary determinant of cell death is the mitochondrial release of Cyt-c. BH3 interacting-domain death agonist, a pro-apoptotic member of the Bcl-2 family, cleaves caspase-8 and increases MOMP, followed by oligomerization of pro-apoptotic proteins Bax/Bak which induce Cyt-c release (52).

The release of Cyt-c is triggered by oxidative stress, facilitates electron transport chain uncoupling, and increases mitochondrial ROS production/levels. Cardiolipin (CL) is an anionic phospholipid that aids Cyt-c anchoring to the inner mitochondrial membrane. The affinity binding with Cyt-c is reduced in oxidative stress due to CL oxidation. CL-bound mitochondrial Cyt-c catalyses CL peroxidation, and this phenomenon can occur at a lower H_2O_2 concentration when bound to CL-containing membranes. Thus, oxidative stress stimulates cell death through the peroxidase activity of Cyt-c and CL peroxidation (52).

3. The role of β-carotene in the modulation of autophagy in puerperal breast inflammation

Mastitis is related to an inadequate intake of vitamin E, selenium, β-carotene, and vitamin A (65). Antioxidants decrease the duration, incidence, and severity of mastitis (23). B-carotene and vitamin A can protect mammary tissues and milk from the destructive effects of free radicals. Food containing vitamin A and β-carotene contributed to a reduced incidence of mastitis during the early dry period in cows (23).

Carotene exhibits a range of important functions, and the numerous benefits of carotenoids are related to their antioxidant and anti-inflammatory effects. Vitamin A and β-carotene trigger the generation of immune cells and prevent the initiation of the fatty acid peroxidation chain reaction (39). Antioxidants directly scavenge free radicals and inhibit the activity of oxidizing enzymes, thus protecting the body from free radicals (23). β-carotene quenches singlet oxygen and neutralizes lipid peroxyl radicals. It has been previously shown that 800 mg/kg body weight of algae Spirulina fusiformis, containing β-carotene and SOD, reduced the levels of almost all oxidative stress biomarkers measured in the study. Serum lipid hydroperoxide levels were reduced by 45%, and leukocyte MDA levels were reduced by 50% (66). β-carotene has a long system of conjugated double bonds with π-electrons delocalized over the length of the polyene chain, providing it with an effective ability to scavenge ROS (67). Studies have shown that β-carotene significantly inhibited intracellular ROS production (30,32,42).

In a study investigating the effects of β-carotene in corneal endothelial cells, a film scaffold incorporated with an appropriate amount of β-carotene showed enhanced initial cell adhesion, proliferation, proper cell morphology, and gene expression compared with a pristine silk fibroin (SF) scaffold. β-carotene in the SF film scaffold enhanced the function of the ATPase pump of corneal endothelial cells (68). In infected tissues, β-carotene decreases the expression of inflammatory mediators, MAPKs, and redox-sensitive transcription factors (69).

β-carotene is widely available in common foodstuffs with broad affordability for the general community. Using β-carotene for postpartum mothers can reduce the occurrence of severe mastitis and maternal and infant morbidity and mortality (32). Although β-carotene and vitamin A serve essential roles in immunity, the latest recommendation from WHO states that vitamin A supplementation for postpartum mothers is not recommended as a public health intervention to prevent maternal and infant morbidity and mortality. Postpartum mothers must obtain a balanced healthy nutritional intake to obtain an adequate amount of various nutrients needed (70,71). Dietary dosage of carotenoids may promote health, but supplementation with high doses may be associated with adverse effects, especially in smokers or subjects exposed to environmental pollutants (72).

β-carotene is abundantly present in green leafy vegetables, carrots, pumpkins, papaya and red palm oil, milk, liver, and fish oil (71). It has been shown that consuming fruit and vegetable sources of β-carotene increases the vitamin A status of lactating women (73). Vitamin A is recommended at 1,300 mcg retinol activity equivalent (RAE) daily. One mcg RAE is equivalent to 2 mcg supplemental β-carotene or 12 mcg dietary β-carotene (74). However, several factors affect the bioavailability and equivalency of vitamin A, such as food processing techniques and the specific dietary intake of an individual (73).

β-carotene plays an essential role in modulating autophagy. In LPS-induced rat intestinal tissue, β-carotene treatment significantly reduced the LPS-mediated phosphorylation of JAK2/STAT3 and NF-κB (59), and significantly suppressed the LPS-induced phosphorylation of JNK and p38 (41) The levels of p-Akt are decreased following LPS treatment, and β-carotene significantly upregulated the phosphorylation of Akt. (41) It also significantly suppressed the ratio of LC3-II/LC3-I, which was upregulated by LPS (41). LC3 consists of LC3-I and LC3-II. LC3-II is positively correlated with the degree of autophagy, and is used as an autophagy marker. LC3-I is present in the cytoplasm where a small segment of the polypeptide is cleaved and is converted into LC3-II (75). β-carotene modulates LPS-induced autophagy and inhibits the activity of the related inflammation pathways (41).

However, another study showed that oral β-carotene supplementation daily for 14 days in male ddY mice (8 wk old) did not alter the ratio of LC3-II to LC3-I in the β-carotene group (76). In H9C2 cell lines, low-dose β-carotene induces autophagy, as shown by a decrease in LC3II and p62 levels. NF-κB protein levels were lowered, and Nrf2 was triggered, but no significant alteration of Nrf1 was observed. A low dose of βcarotene stimulates cell viability by suppressing the apoptotic signals carried by caspase 3 and 9. Furthermore, a low dose of β-carotene increases cell viability through autophagy stimulation, inhibition of pro-inflammatory factors, and suppression of apoptosis (36).

Treatment of SAOs and Caco-2, cell lines derived from a human osteosarcoma and colon adenocarcinoma, respectively, using 200-400 μg/ml carotene extract nanoemulsion (CEN) triggered autophagy, based on the increase of autophagosome formation, increased expression of LC3-II, modulation of autophagic flux, increased phosphorylation, and an increase in the levels of the active form of AMPK kinase $pAMPK^{Thr172}$,

which activates AMPK and induces autophagy (33). β-carotene is the major carotenoid in CEN (33). However, treatment with the same dose of β-carotene alone did not show the same effect as when combined in the CEN, indicating a dose-dependent effect or a synergism of different components in CEN (33).

The PI3K/Akt/mTOR signaling pathway is important in cell survival and autophagy (77). Inhibition of this signaling pathway induces autophagy. Consistent with previous studies, β-carotene treatment was also shown to decrease Advanced Glycation End products (AGE)-induced elevation of the LC3II/LC3I ratio and the number of LC3-labeled puncta. The protective effect of β-carotene in AGE-induced H9c2 cells was achieved through the activation of the PI3K/Akt/mTOR signaling pathway (42). Fig. 2 summarizes the pathways affected by β-carotene as an autophagy modulator.

β-carotene shows promising potential as an autophagy modulator in increasing mammary gland cell survival and maintaining breastmilk production. Further research is required to understand the role of β-carotene in puerperal breast inflammation management.

4. Conclusion

Puerperal breast inflammation can cause result in cessation of breastfeeding due to mammary gland damage and a decrease in milk production. Excessive ROS levels in puerperal breast inflammation results in oxidative damage and induces improper autophagy. β-carotene has ROS-scavenging activity and the ability to modulate autophagy through the suppression of JAK2/STAT3, NF-κB, JNK, and p38 phosphorylation, suppressing the ratio of LC3-II/LC3-I and upregulating the phosphorylation of Akt. Further studies are required to conclude the full potential of β-carotene in puerperal breast inflammation.

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Availability of data and materials

Not applicable.

Authors' contributions

STH, RL, JWG, and ER performed the literature search and assisted in drafting and revising the manuscript. All authors have read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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