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Application of plant-derived exosome-like nanovesicles in the treatment of bone diseases

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Abstract: Plant-derived exosome-like nanovesicles (PELNs) are secreted by plant cells and play crucial roles in plant metabolism and immune defense. They can also regulate physiological activities across species, offering broad application potential. This review provides a comprehensive summary of recent advancements in PELNs, covering their biogenesis pathways, components, extraction, separation and identification methods, as well as their applications in skeletal system diseases. Finally, the review discusses the challenges faced in the field and suggests future research directions, offering valuable insights for further studies.

Extracellular vesicles (EVs) have been a major focus of scientific research since their discovery. Early studies primarily concentrated on EVs derived from animal cells[1]. However, the clinical application of animal cell-derived EVs is limited due to low yields, complex extraction methods, and the potential to trigger immune responses[2]. As a result, researchers have shifted their attention to plant-derived exosome-like nanovesicles (PELNs).

Although PELNs were discovered before EVs, they did not gain widespread attention until 2009, when researchers isolated PELNs (20-200 nm in diameter) from sunflowers and identified their potential role in intercellular communication[3]. Compared to animal cell-derived EVs, PELNs offer several advantages, including lower immunogenicity, higher biocompatibility, fewer side effects, and more efficient extraction. As a result, PELNs have shown growing promise in biomedicine, particularly for treating skeletal system diseases.

1. Biogenesis of PELNs

The molecular mechanism of PELN biogenesis is not yet fully understood, but it is generally believed that the multivesicular body (MVB) pathway is the primary mechanism[4]. Studies have shown that when plants are attacked by fungi or other pathogens, the cytoplasmic membrane invaginates twice to form multivesicular bodies[5]. These bodies contain intraluminal vesicles (ILVs), which carry various biomolecules such as lipids, DNA, RNA, and proteins, facilitated by endosomal sorting complexes[6]. The MVBs then either fuse with lysosomes for degradation or with the plasma

membrane to release ILVs as exosomes into the extracellular environment[7].

In addition to the MVB pathway, the exocyst-positive organelle (EXPO) pathway also contributes to PELN biogenesis. This pathway is marked by the formation of EXPO structures, which fuse with the plasma membrane to release vesicles into the external environment[8]. Unlike the MVB pathway, the EXPO pathway is not affected by secretory pathway inhibitors, potentially due to the action of S-adenosylmethionine synthetase 2 (SAMS2)[9]. Additionally, the vacuolar pathway plays a crucial role in plant resistance to fungal infections. In this process, MVBs fuse with the vacuole to release ILVs, which are then transported to the plasma membrane and released into the extracellular space[10].

2. Composition of PELNs

PELN lipids, primarily composed of glycerides and phospholipids, differ from those in EVs[11]. Studies have shown significant variation in the lipid composition of plant-derived PELNs. For example, ginger-derived PELNs mainly contain phosphatidic acid, dilactosyl diacylglycerol, and mono-galactosyl monoacylglycerol[12], while PELNs from Panax notoginseng are rich in phosphatidic acid[13]. Phosphatidic acid regulates vesicle formation, morphology, and fusion[14]. In ginger-derived PELNs, it interacts with heme-binding protein 35 from Porphyromonas gingivalis, inhibiting its growth and enhancing their therapeutic effect against chronic periodontitis[9]. These findings suggest that lipids in PELNs influence both their formation and biological functions.

PELN proteins are primarily intracellular, including actin and proteolytic enzymes[16]. These proteins play key roles in cell function, intercellular communication, and pathogen resistance, particularly in cell wall remodeling[4].

Additionally, PELNs contain RNA, which is essential for gene regulation and intercellular communication[17]. Zhang et al. identified hundreds of miRNAs in ginger-derived PELNs that may regulate gene expression by binding to the 3' untranslated region of target cells[18]. Another study analyzing the miRNA profiles of PELNs from 11 different fruits and vegetables found that certain miRNAs regulate inflammatory and cancer-related genes in vitro[19].

3. Extraction and separation of PELNs

3.1. Ultracentrifugation

Ultracentrifugation, the earliest method for isolating extracellular vesicles, separates PELNs based on differences in density and particle size. The process involves differential centrifugation and density gradient centrifugation.

In differential centrifugation, samples are first centrifuged at low speed (2000–3000 ×g, 20–30 min) to remove large debris. Next, high-speed centrifugation (10,000–20,000 ×g, 60 min) removes small organelles. PELNs remain in the supernatant, while non-target particles settle. Ultracentrifugation (100,000–200,000 ×g, 1–2 hours) precipitates PELNs, which are then resuspended in PBS. Although cost-effective, this method often results in contamination with proteins, nucleic acids, and other vesicles, leading to lower purity[20].

Density gradient centrifugation separates PELNs based on differences in sedimentation coefficients by creating density zones in a gradient. Iodixanol and sucrose are commonly used as gradient media. This method requires precise control of centrifugation speed and time, and the yields are typically low[21, 22].

A combined approach using differential centrifugation and sucrose density gradient centrifugation is commonly employed. For example, Li et al. extracted crude PELNs from Panax ginseng using differential centrifugation, followed by further purification with sucrose density gradient centrifugation. The final PELNs, approximately 150 nm in diameter, exhibited a typical cup-like

structure, with significantly improved purity and preserved structural integrity, providing more reliable samples for research[13, 23].

3.2. Polymer precipitation method

The polymer precipitation method relies on using polymers, typically polyethylene glycol (PEG), to bind with non-water-soluble proteins and lipids, reducing the solubility of PELNs. The solution is then centrifuged at high speed at 4 ℃, and the precipitate is collected and resuspended in PBS buffer[24]. Compared to ultracentrifugation, this method offers advantages such as simplicity, shorter processing time, and lower cost. However, PELNs obtained by this method often have lower purity and may contain additional proteins and impurities[25]. Studies have shown that the recovery and purity of PELNs using polymer precipitation are influenced by pH and operating conditions. At low pH, recovery of ginger-derived PELNs can increase by 4-5 times[26]. Additionally, reducing PEG concentration and shortening precipitation time can improve purity and reduce impurities[27].

3.3. Size exclusion chromatography and other methods

Size exclusion chromatography (SEC) is a molecular size-based separation technique that effectively isolates exosomes by matching particle size with the pore size of resin beads in the column[25]. SEC is widely used for isolating PELNs due to its simplicity, efficiency, high purity, and ability to preserve exosome structure and function[22]. Common packing materials include dextran, agarose, and polyacrylamide[21]. SEC is often combined with other methods for improved isolation. For example, Ramesh Bokka et al. combined ultracentrifugation with SEC to isolate tomato-derived PELNs, effectively removing proteins and small molecules, which improved exosome yield[28]. However, SEC may retain hyaluronic acid, which plays a key role in immune regulation and may affect exosome function[29].

With advances in PELNs research, new extraction and separation methods are emerging. For instance, electrophoresis with dialysis bags has been used to rapidly isolate PELNs from lemon juice, producing vesicles similar in size and number to those obtained by ultracentrifugation[30]. Additionally, the fast capillary channel polymer fiber spinning tip method has been applied to isolate PELNs from various fruits and vegetables with high efficiency, yield, and purity[31].

4. Methods for identification of PELNs

The identification of PELNs involves parameters such as morphology, particle size, and surface charge. Transmission electron microscopy (TEM), scanning electron microscopy (SEM), and cryo-electron microscopy are commonly used to examine their morphology. While TEM offers high resolution, both TEM and SEM require sample dehydration and fixation, often resulting in a cup- or saucer-shaped structure[32]. Cryo-electron microscopy, however, preserves the true spherical morphology without the need for dehydration or fixation[33].

Particle size and surface charge are measured by nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS). NTA tracks individual PELN movement, using Einstein's equations to calculate concentration and size[34]. DLS provides an average size but lacks resolution and cannot measure concentration, leading to its replacement by NTA for PELN size characterization. NTA produces more stable results, less affected by large particle scattering[35]. PELNs typically range from 40 to 200 nm, with a negative zeta potential, usually between neutral and -30 mV. Nanoparticles with a zeta potential between -30 mV and +30 mV are generally stable[15].

Improper extraction can introduce impurities, affecting NTA and DLS results. Recently, local surface plasmon resonance (LSPR) technology has shown high sensitivity in detecting exosomes[36].

Atomic force microscopy and LSPR are also gaining attention for liquid biopsy applications, particularly in diagnosing malignant tumors like glioblastoma[37].

5. Application of PELNs in skeletal system diseases

5.1. Administration methods

In in vivo studies of PELNs, the route of administration plays a critical role in their efficacy and safety. The two main administration methods are oral and injectable. Oral administration is safe and convenient. A 2023 study found that PELNs derived from yams were highly resistant to digestive enzymes in simulated gastric and small intestinal fluids, suggesting that their gastrointestinal tolerance may be related to their lipid composition[38].

In contrast, injectable delivery requires higher purity and sterility. Seo et al. investigated the inhibitory effect of ginsenoside-derived PELNs on bone resorption in osteoporotic mice via subcutaneous injection[39], while Kim et al. examined the distribution of ginsenoside-derived PELNs in mice and their effect on glioma through intravenous injection[40]. Another study compared the effects of different administration routes for camellia-derived PELNs in mice. The results showed that intravenous injection led to higher levels of alanine aminotransferase, complement C3, and aspartate aminotransferase, indicating potential liver damage compared to oral administration[41].

5.2. Osteoporosis

Osteoporosis is a bone disease characterized by decreased bone mineral density due to an imbalance between bone resorption and formation. Anti-osteoporosis drugs aim to reduce fracture risk by promoting bone formation or inhibiting resorption. Common treatments such as estrogen, calcitonin, and bisphosphonates can restore bone density but may cause serious side effects with long-term use and have limited efficacy[42]. This has led to increased interest in natural products as alternative treatments.

In promoting bone formation, Yue Cao et al. found that Morinda officinalis-derived PELNs enhance MC3T3-E1 cell proliferation via the MAPK signaling pathway, improving bone formation[43]. Hwang JH et al. showed that yam-derived PELNs promote osteoblast proliferation, differentiation, and mineralization by activating the BMP-2/p-p38/Runx2 pathway, increasing bone mineral density and improving osteoporosis in mice[38]. Similarly, apple-derived PELNs promote MC3T3-E1 cell proliferation and mineralization via the BMP2/Smad1 pathway[44]. Qianxin Liang et al. observed that drynaria-derived PELNs enhance mesenchymal stem cell proliferation and differentiation towards osteogenesis[45]. Pueraria-derived PELNs also alleviate osteoporosis by regulating trimethylamine-N-oxide, a gut microbiota metabolite[46].

In inhibiting bone resorption, Seo K et al. found that ginseng-derived PELNs inhibit RANKL-induced signaling pathways (IκBα, c-JUN N-terminal kinase, and ERK), suppressing osteoclast differentiation[43].

5.3. Osteoarthritis

Osteoarthritis (OA) is a common degenerative joint disease often associated with aging, mechanical damage, genetic factors, and obesity. It is characterized by articular cartilage degeneration, synovial inflammation, osteophyte formation, and changes in periarticular structures[47]. Chronic pain and joint dysfunction caused by OA significantly affect patients' quality of life. Current treatments mainly focus on symptom management and joint replacement, but they do not address the underlying mechanisms of the disease. Long-term drug treatments may also have serious side effects. Therefore,

exploring safer and more effective treatment options has become a key area of research. In this context, PELNs have gained attention as a promising new approach for treating osteoarthritis[48].

One study found that tomato-derived PELNs enhanced the expression of chondrocyte markers ACAN, SOX9, and COMP, as well as key proteins COL2 and COLXI. This promoted the differentiation of human adipose-derived MSCs into chondrocytes, suggesting the potential of tomato-derived PELNs for treating osteoarthritis[49].

6. Summary and Prospect

As research on plant exosome-like nanoparticles (PELNs) progresses, they are emerging as promising therapeutic agents due to their biocompatibility, wide availability, and safety. However, several challenges remain in their development.

Firstly, many studies overlook the impact of plant origin and seasonal variations on the consistency and quality of PELNs, limiting their broader application. Recent advances in plant tissue culture techniques show potential for providing a stable supply of PELNs, but further efforts are needed to standardize PELNs from various plant sources and developmental stages. Improved tissue culture methods should ensure high-quality, large-scale production with rigorous quality control.

Secondly, the extraction and separation methods for PELNs require optimization. Current techniques, such as ultracentrifugation, polymer precipitation, and size-exclusion chromatography, each have advantages and limitations, leading to variability in purity and yield. Developing more efficient and reproducible methods is essential for both research and clinical applications. Additionally, unlike animal-derived EVs, PELNs lack established protein markers. Future research should focus on proteomic analysis to identify reliable plant exosome markers for better cross-study validation.

Lastly, while PELNs show promise in treating skeletal diseases—such as promoting bone formation and repairing cartilage—research on conditions like osteoarthritis and degenerative spine diseases remains limited. PELNs offer superior safety, biocompatibility, and stability compared to animal-derived EVs as drug carriers. However, their use in drug delivery for skeletal diseases is still in the early stages, with most studies confined to in vitro and animal models. Large-scale clinical trials are needed to confirm their therapeutic efficacy.

In conclusion, PELNs hold significant potential as a novel biomaterial with broad applications. Future research should focus on expanding their use in skeletal diseases, optimizing extraction and characterization techniques, understanding their mechanisms in disease treatment, and addressing challenges in clinical translation. Collaborative research could pave the way for innovative solutions in orthopedic disease prevention and treatment.

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