ABSTRACT

Bovine Milk Exosomes for the Treatment of Melanoma

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Introduction

Cancer is a leading cause of death worldwide, and skin cancer, especially melanoma, has seen a significant increase in both new cases and mortality in the past decade. In 2020, there were an estimated 325000 new cases of melanoma and 5700 deaths. By 2040, new cases are projected to rise to 510000 and deaths to 96000. During the later stages of melanoma, the cancer cells metastasize to other parts of the body, especially the lungs, via the vascular or lymphatic system. Unfortunately, there are limited therapies available for treating metastatic melanoma, aside from surgery. Many chemotherapeutic drugs have been developed to treat melanoma, but most of them have adverse effects. The development of these drugs is extensive, and there is a need for a cost-effective drug or drug delivery system with fewer or no adverse effects. Drug development is a challenging process, as finding the optimal compound from lead is difficult, and many drugs fail in the preclinical stage due to safety and efficacy issues. Drug repurposing involves finding new uses for existing drugs, which can significantly reduce the time and cost of developing effective treatments for skin cancer. Repurposed drugs offer several advantages, including readily available data from clinical trials, information about their chemical composition, and potential toxicity, all of which can expedite their use in clinical trials. The medicinal properties of various phytochemicals, such as alkaloids, terpenoids, flavonoids, polyphenols, and their analogs, have been thoroughly researched. Despite their high potential for repurposing, many of these phytochemicals have been overlooked in this context. Several studies have shown the promise of repurposed phytochemicals in treating different types of advanced skin tumors. The repurposed phytochemicals from various classes, such as phenolic compounds, polyphenols, alkaloids, and terpenoids, have shown great promise in combating a variety of skin cancers. They target different hallmarks such as glutathione attenuation, activation of apoptosis, cell cycle arrest, generation of reactive oxygen species (ROS), and inhibition of diverse signaling pathways. Flavonoids, which are plant-derived polyphenolic secondary metabolites, have garnered substantial attention for their potential as therapeutic agents. This heightened interest is attributed to their versatile mechanisms of action, cost-effectiveness, and minimal adverse effects. Dihydroartemisinin (DHA) is a semi-synthetic derivative of artemisinin (AT) derived from Artemisia annua L, usually employed in the treatment of malaria. Recent studies have demonstrated the potential of DHA as an anticancer agent in various malignancies such as melanoma, prostate, breast, colon, and ovarian cancer. DHA exerts its antineoplastic effects through diverse mechanisms, including the inhibition of TGF-β signaling, which activates cancer-associated fibroblasts, downregulation of

proliferative markers (PCNA, Cyclin E, and Cyclin D1), induction of apoptosis by upregulating BAX and caspase-3 expression, induction of cell cycle arrest, and attenuation of cell migration and angiogenesis via the inhibition of matrix metalloproteases (MMPs) and vascular endothelial growth factor receptor-2 (VEGFR2). Similarly, Hesperidin (HES), a flavonoid present in citrus fruits, demonstrates diverse pharmacological activities, encompassing cancer treatment, anti-inflammatory effects, and relief from conditions such as haemorrhoids and varicose veins. Hesperidin's anti-cancer properties are evidenced through intrinsic and extrinsic apoptosis pathways, the generation of reactive oxygen species, and the modulation of various anti-inflammatory factors such as TNF-α, IL-1β, COX-2, and iNOS. These properties have been substantiated by numerous *in vitro* and in vivo studies involving various cancer types. Despite showing a commendable anticancer and other pharmacological activities by DHA, and HES, their therapeutic efficacy faces limitations due to poor oral bioavailability, extremely low water solubility, and a short half-life. Further, DHA was found to be administered in multiple doses to maintain the therapeutic window due to the short half-life, which might lead to dose-dependent side effects. To address these issues, scientists have investigated different delivery systems to improve solubility, bioavailability, and reduce toxicity. These systems include inorganic gold nanoparticles, PLGA nanoparticles, polymeric micelles, self-assembled nanoparticles, metal-organic frameworks, magnetic nanoparticles, and liposomes. However, each artificially created delivery system has its limitations, which could potentially be addressed by the development of naturally derived delivery systems. Exosomes (Exo), which are biologically derived lipid-based nanoparticles with a diameter of less than 200 nm, demonstrate the capacity to transport a diverse array of cargo, encompassing DNA, RNA, proteins, miRNA, and other biomolecules. These membrane-bound extracellular vesicles are discharged from all cell types following the fusion of an intermediate endocytic compartment known as the multivesicular body (MVB) with the plasma membrane. Found in various bodily fluids, including milk, exosomes play a pivotal role in mediating intercellular communication and engaging in numerous physiological processes. Their surface is adorned with CD44 proteins, facilitating prolonged circulation and mitigating drug degradation by evading rapid clearance. Possessing a biocompatible nature, exosomes exhibit compatibility with biological systems and can traverse diverse physiological barriers, such as the blood-brain barrier (BBB). Their non-immunogenicity, coupled with low production costs and minimal to no toxicity, collectively position them as a promising candidate for drug delivery applications. In the current study, we aim to deliver DHA, and HES through bovine milk exosomes to improve its

pharmaceutical attributes and enhance therapeutic efficacy for the effective treatment of melanoma.

Objectives

The objectives of the present research work include:

- Isolation, characterization, *in-vitro* and *in-vivo* investigation of dihydroartemisinin-loaded bovine milk exosomes
- Isolation, characterization, *in-vitro* and *in-vivo* investigation of hesperidin- loaded bovine milk exosomes

Methodology

Exosomes were isolated from bovine milk using a differential centrifugation process and drugs (DHA, and HES) were loaded into the isolated exosomes using a sonication technique to prepare DHA-Exo, and HES-Exo. The developed exosomes were then characterized for particle size, polydispersity, zeta potential, morphology, %entrapment efficiency, % drug loading, and *in vitro* drug release profile. Further, the surface phenomena were analyzed using HR X-ray diffraction. Further, to evaluate the anticancer efficacy of the pure drugs, and the developed exosomes in the cellular system, various cell culture assays were performed in B16F10 cells, where we performed the MTT assay, qualitative and quantitative cellular uptake assay, DNA fragmentation assay, reactive oxygen species (ROS) assay, mitochondrial membrane potential (MMP) assay, colony formation assay, wound healing assay, and transwell migration assay. Moreover, to analyze the biocompatibility of the developed formulations, MTT assay was performed in HEK – 293 cells. Further, the pharmacokinetic study was performed in healthy female Sprague−Dawley rats to analyze the prevalence of drugs and developed exosomes in systemic circulation. The anticancer activity of the developed exosomes was evaluated in B16F10-induced melanoma Swiss Albino mice. The anticancer study was continued for 21 days, after which they were sacrificed for analyzing anticancer efficacy by evaluating tumor volume, tumor weight, body weight of tumor-bearing rat, and tumor inhibition rate. The anticancer activity was also analyzed using hematological analysis, biochemical analysis, and histopathology.

Summary & Conclusion

The exosomes were successfully isolated from bovine milk using a differential centrifugation process and drugs were loaded into the exosomes using the sonication method where the ratio

of drug: exosomes were 1:9 (i.e. 1 part of drug and 9 part of isolated exosomes). The particle size, PDI, and morphology of the developed exosomes were found to be <150nm, < 0.2, and spherical respectively, as suitable for oral delivery. The developed drug-loaded exosomes showed an increased negative surface charge as compared to naive exosomes, inferring their stability. Further, both the DHA-Exo, and HES-Exo exhibited a maximum % entrapment efficiency and % drug loading of ~80%, and ~20% respectively. Further, the surface interaction of exosomes and the drugs was revealed using HR-XRD. The *in vitro* drug release study demonstrated a biphasic release profile with an initial burst release followed by a sustained release of drugs in both pH 7.4 (systemic circulation), and pH 5.5 (tumor microenvironment), with a higher release profile observed at pH 5.5, which may be due to the rupture of the exosomal membrane, facilitated by proteins and peptidoglycans, leading to increased drug release. The *in vitro* studies conducted on B16F10 cell lines demonstrated higher cytotoxicity of DHA-Exo, and HES-Exo, compared to free DHA, and HES, as supported by enhanced cellular uptake validated through coumarin-6-loaded exosomes. This superior cytotoxicity was further evidenced by DNA fragmentation, increased generation of free radicals (ROS), loss of mitochondrial membrane potential, and effective inhibition of colony formation. The antimetastatic properties of DHA-Exo, and HES-Exo were confirmed through wound healing and transwell migration assays. Oral pharmacokinetics studies revealed a remarkable increase of approximately 2.5 times in oral bioavailability and half-life of drugs when loaded into exosomes. Subsequent in vivo experiments utilizing a B16F10-induced melanoma model in Swiss mice established that DHA-Exo and HES-Exo exhibited superior anticancer activity compared to DHA, and HES after oral administration. Importantly, no biochemical, hematological, or histological toxicities were observed in tumor-bearing mice treated with DHA-Exo, and HES-Exo. These findings suggest that exosomes loaded with DHA, and HES represent a promising nanocarrier strategy to enhance the therapeutic effectiveness of DHA, and HES in melanoma treatment.