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REVIEW

REMEDIAL APPLICATIONS OF EXOSOMES IN CANCER, INFECTIONS AND DIABETES

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Abstract: Different cell types under normal and diseased states constantly secrete numerous membrane vesicles including exosomes into extracellular space wkich can be isolated from various biological fluids and cell culture supernatants. Exosomal diameter ranges between 40-100 nm. In current research, exosomes are being exploited as biomarkers for pathological diagnosis and potential remedy against various disease conditions such as infections and autoimmune disorders. In cancer immunotherapy, exosomes have promisingly been employed due to the identical immunogenic antigens of exosomes produced by neoplastic cells and the originating tissues. Antigen-specific T-cell activation and immunomodulatory activity is observed to be performed by exosomes isolated from tumor and dendritic cells (DCs). However, more research is still required to uncover the realistic uses of exosomes, particularly as drug delivery tool in autoimmune diseases, cancers and diabetes.

Keywords: drug delivery, exosomes, isolation, infection, neovascularization, remedy

Exosomes are extracellular nanobodies (40-100 nm diameter) consisting of cytoplasm and lipid bilayer as core and coat, respectively, and transmembrane proteins. All normal and diseased cell types like platelets, B- and T-cells, epithelial cells, dendritic cells (DCs), and mast cells secrete these membrane vesicles (1, 2) into the extracellular environment i.e., biological fluids like serum, urine, breast milk, cerebrospinal fluid, saliva, malignant effusions, and bronchoalveolar lavage fluid (4-14). Exosomes develop from multivesicular bodies, called endosomes, through the formation of intracellular buds followed by their fusion with cell membrane resulting in the secretion of these internal vesicles (Fig. 1).

Currently, a lot of research is in progress to explore biological activity of exosomes. It has been examined that exosomes are engaged in the swap of hereditary characteristics by mediating the intercellular communication. Their shielding effect against any stress has also been observed (1-3). To diagnose diseases, the role of exosomes as biomarkers is promisingly being exploited (12, 13, 15-20).

As a result of various analytical studies (21-23), exosomes originated from different cell types (21-33) have been shown to be composed of common as well as cell type specific proteins which, apparently, seem to be vital for the synthesis of exosomes and physiological role of the parent cell, respectively. The common set of proteins in exosomes comprises of cytoplasmic proteins, like membrane proteins (tetraspanins e.g., CD80 and CD86), tubulin, trimeric G proteins, actin, and the heat shock proteins (Hsp70 and Hsp90) (37). These ubiquitous proteins have been proposed to play their role in the linking of cells and their activation, propagation and presentation of antigen. Cell-type specific proteins are presumably involved in specialized functionality of exosomes depending upon the parent cells, such as DCs-origin exosomes possess major histocompatibility complex (MHC, class I and II) and express co-stimulatory protein molecules, such as CD54 and CD80 (also called ICAM-1 and B7-1, respectively) which are involved in T-cell stimulation (15, 22, 24, 32-35). The urine origin exosomes have aquaporin-2, which might play a crucial role in the diagnosis of renal diseases (36). Citrullinated proteins, such as fibrin α- and β-chains, are involved in autoimmune responses and have been found in exosomes collected from the supernatant of synovial fluid (35).

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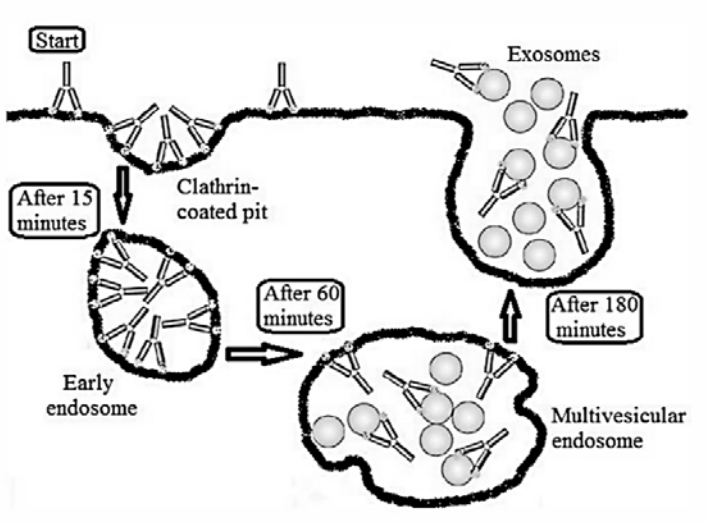

Figure 1. Developmental stages of exosomes (10)

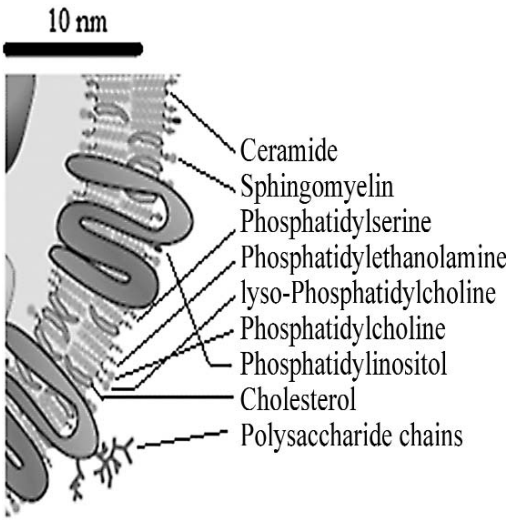

Figure 2. Membrane structure of exosomes illustrating its resemblance to cell membrane (40)

The analyses of exosomes originated from different cell types have elaborated lipids as characteristics of parent cell (37-40), such as mast cell-derived exosomes possessing lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, cholesterol and diglyceride (Fig. 2) (40). These ubiquitous lipids are present in different ratios in exosomes collected from various cell types, such as B cell-derived exosomes which show higher ratio of cholesterol/phospholipid as compared to that of mast cell derived exosomes (39).

Exosomes are separated from supernatants of cell culture. Of all components in a biological fluid, there is a small percentage of exosomes. The density and size of exosomes is examined as 1.13-1.21 g/mL and 40-100 nm, respectively. Since the exosomes contain cell type specific proteins, the isolation approaches depend upon their density, size, and other biochemical characteristics (13). Generally, exosomes are fractionated through consecutive centrifugation process of biological fluids and culture supernatants for the removal of waste cells and cell debris (Fig. 3). The advantage of ultracentrifugation process includes the purification of exosomes from cellular fragments such as proteins depending upon their differential densities (41). However, some negative aspects of ultracentrifugation have been observed which include: (i) achievement of heterogeneous mixture of exosomes and other micro-size vesicles due to identical size, thus this technique fails to fulfill the criteria of current Good Laboratory Practice (cGLP) (42), (ii) rupturing of exosomes due to the application of extreme centrifugation force (43), and (iii) aggregation of exosomes with other cellular entities such as cell debris resulting in the development of a pellet which hinder the purification process, and thus inconsistent quantity of exosomes is recovered (5-25% of initial concentration) (44). Ultrafiltration technique is an excellent approach to purify cGMP grade exosomes from bulky (greater than one liter) medium employing pumps and cartridges. Additionally, another modality involves the isolation of exosomes on the basis of their biochemistry. Practically, magnetic beads are coated with monoclonal antibody particular for a protein recognized to be present on the membrane of exosomes, such as HER2-expressing tumor exo-

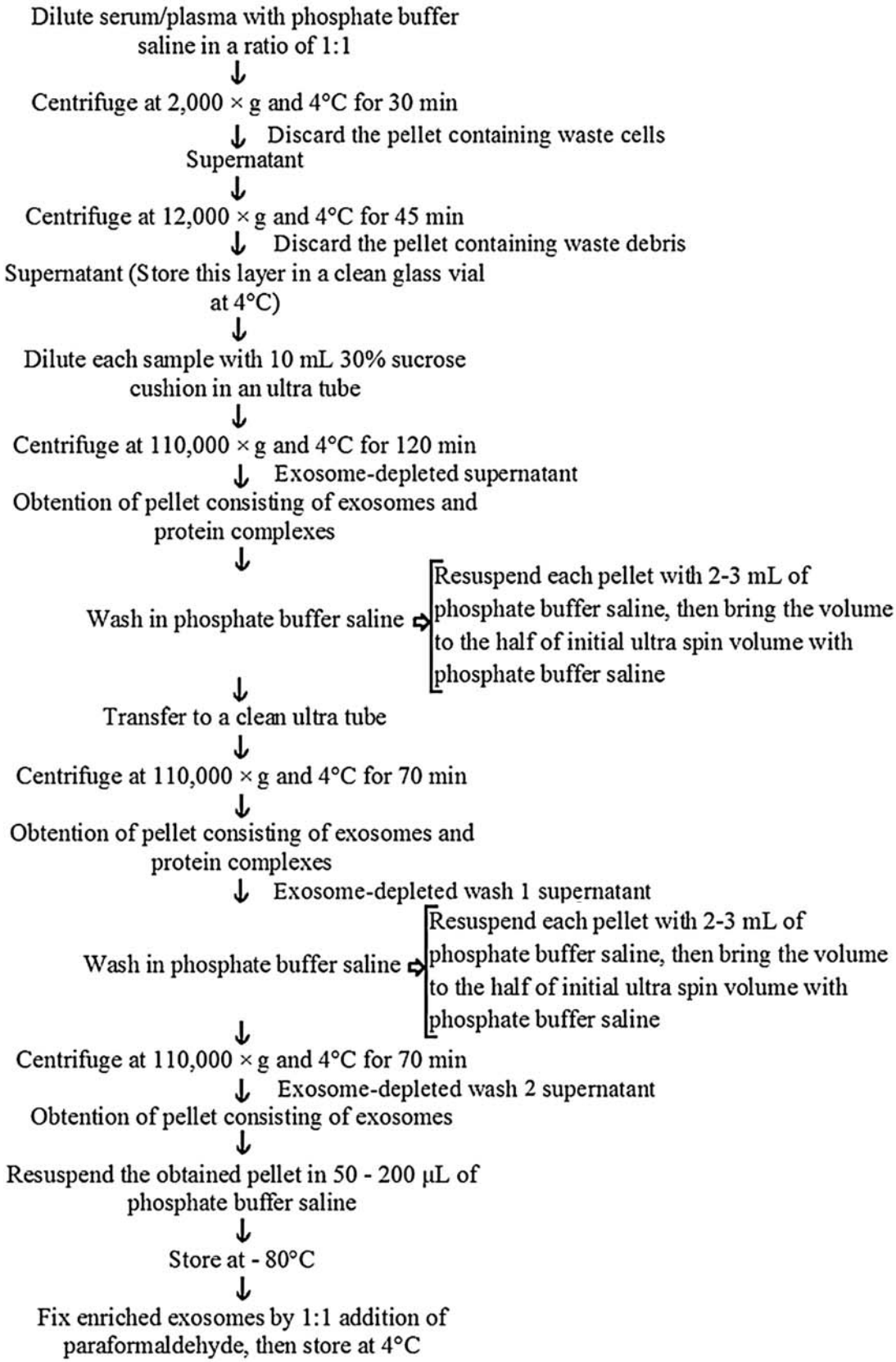

Figure 3. General ultracentrifugation method for isolation of exosomes from serum/plasma (49)

somes can be collected from the supernatant layer of breast adenocarcinoma cell lines using magnetic beads coated with antibodies against tumor-specific proteins (41, 43, 49).

LITERATURE SEARCH METHODOLOGY

During previous twelve years, massive attention of researchers in exosomes was observed. From year 2000 to 2012, approximately 1000 publications were reported in PubMed indexed journals. The literature search was made using different keywords, such as exosomes, exosomes as drug delivery tool, or therapeutic exosomes followed by manual verification of found articles by understanding their title and summary for making certain that the publication referred exosomes without duplication.

RESULTS AND DISCUSSION

In cancer immunotherapy, exosomes have promisingly been employed due to the identical immunogenic antigens of exosomes produced by neoplastic cells and the originating tissues, such as immunogenic antigens MelanA/Mart-1 and gp100 are expressed in the exosomes derived from melanoma while immunogenic antigens CEA and HER2 are present on the exosomes obtained from colon carcinoma tissues. Microvesicles obtained from plasma of cancer patients also express these immunogenic antigens demonstrating the source of cancer of these microvesicles (14, 50). Tumor inhibition has been observed to occur after *in vivo* administration of exosomes holding tumor antigens, the mode of action of these antigens may involve the stimulation of $CD4^+$ and $CD8^+$ T cells and ultimately induction of T-cell activity (51-53). Exosomes isolated from dendritic cell as well as from *in vitro* cultured antigen presenting cells (APC) also express antigens derived from tumor, and these antigens inhibit the tumor growth in mouse (14). There are many similar features between human and mouse, but their immune systems are different from each other (such as canonical Th17 differentiation signature e.g., IL17A, pro-inflammatory response and $CD4^+$ T cell activation of human is dissimilar from mice) due to which very vigilant research is needed to translate these results from mouse to human.

Delcayre and Le Pecq (54) and Viaud et al. (55) developed patient specific doxosomes by loading dendritic cell-derived exosomes with tumor antigen-derived peptides for melanoma and evaluated its therapeutic efficacy in human cancer during phase I clinical tests. Doxosomes initiated immune response (both innate and adaptive) in cancer patients exhibiting their immunotherapy as a safe remedial approach (54, 55). Dai et al. (56) studied ascites-derived exosomes with tumor antigen-derived peptides alone and in combination with

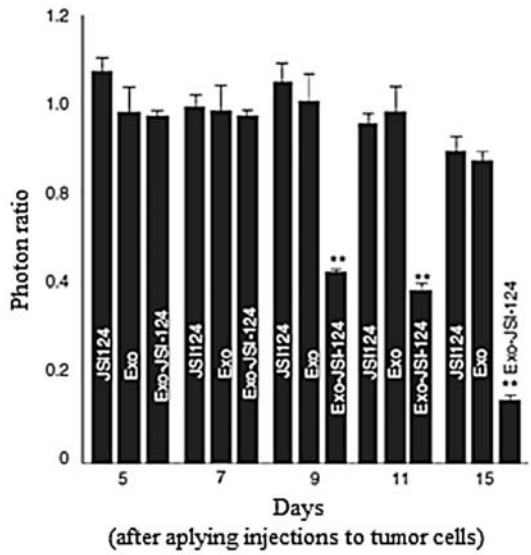

Figure 4. Prevention of brain tumor growth after injecting exosomal JSI124. (Photon ratio is calculated by dividing the number of photons accumulated for treated mice by the number of photons accumulated for untreated mice at different imaging time points using Living Image 2.50 software, Xenogen®) (56)

GM-CSF as a safe cancer vaccine in colorectal cancer in human cancer during phase I clinical trials (56). Regardless of excessive production of tumor-derived exosomes, their prospective antitumor activity is still ambiguous (53). Viaud et al. (55) also observed enhanced tumor growth after *in vivo* administration of tumor-derived exosomes revealing its immunosuppressive nature (55). Therefore, tumor-derived exosomes should efficiently be characterized to understand their influence on tumor pathogenesis for further advancement in their remedial application in cancer. Zhuang et al. (57) proposed a new modality providing a non-parenteral remedial method for the treatment of brain inflammation. They prepared curcumin-encapsulated exosome and JSI124-loaded exosome (JSI124 is a signal transducer and activator of transcription 3 inhibitor) and delivered the products to microglial cells of two different groups of mice through their intranasal pathway. The results revealed that both groups of mice remained safe from lipopolysaccharide-induced inflammation of brain. The study showed considerably delayed growth of brain tumor in the GL26 tumor model (Fig. 4). This modality exhibited swift delivery of exosomal microcapsules to the brain and then selective uptake of these microcapsules by microglial cells resulting in their apoptosis (56).

Exosomes have been found to be safe vaccine candidates in some infectious condition, e.g., toxoplasmosis, diphtheria, tuberculosis and atypical severe acute respiratory disorder. *Toxoplasma gondii* is an obligate intracellular parasitic protozoan which causes toxoplasmosis, a disease that passes from animals to human. Previous publications (57, 58) describe a difficult procedure to acquire large amount of DC appropriate for vaccination, where DC expressed with *T. gondii* antigens (TAg) transfer to healthy mice and provoke defense against a dangerous oral confront of *T. gondii*. Dendritic cell-derived exosomes are found to be an alternate of dendritic cell-based vaccines to provoke defensive immune responses, such as defensive immune responses are provoked by exosomes isolated from SRDC (CD8α + CD4- DC cell line) pulsed *in vitro* with *Toxoplasma gondii*-derived antigens (termed as Exo-TAg) for *T. gondii* in mice resulting in the survival of allogeneic mice, while some brain cysts were observed in syngeneic mice (59). The remedial application of exosomes has also been studied in diphtheria, a disease caused by diphtheria toxin (DT). After being injected, the mice with murine bone marrow derived DCs pulsed *in vitro* with integral DT-released exosomes, immunoglobulin G (IgG)2b and IgG2a responses are provoked against DT (60). An augmented production of exosomes homed with *M. tuberculosis* peptide-MHC-II complexes, has been observed in tuberculosis, an infection caused by *Mycobacterium tuberculosis*, resulting in T-cell mediated responses (61). The remedial application of exosomes has also been studied in a lethal respiratory infection caused by SARS-associated coronavirus by developing SARS-S exosomal vaccine which provokes immune response through the neutralization of antibody titres (62). An exosomal vaccine can also be employed to manage allergic conditions. Mice have been protected from allergic sensitization by using exosomes separated from the bronchoalveolar irrigation of tolerized-mice exposed to olive pollen (63). The naïve people can be protected against ovalbuman (OVA) if they have previously been injected with exosome-loaded serum procured from the experimental animals nourished with OVA (64). Though, detailed biological study of breast milk-derived exosomes (BMDE) and its influence on allergic reactions is still in progress, however BMDE contain immune-regulating molecules e.g., heat-shock proteins (Hsps), MUC-1, MHC and CD86, which inhibit the production of IL-2 and IFN-γ (65).

Autoimmune disorders in animal models have also been treated using exosomes. For example, delayed-type hypersensitivity inflammation has effectively been suppressed using dendritic cell-derived exosomes expressing recombinant IL-4 to manage collagen-induced arthritic condition through the modulation of T-cell and APC activity (66). Moreover, arthritic condition in mice was also promisingly suppressed by exosomal vaccine originated from IL-10 and FasL-modified DC (67). Bowel inflammation is also treated using exosomes from TGF-β1-modified DC (68).

Being safe, small size, lipid bilayer structure and natural vesicles, exosomes can easily cross biological membranes, so their application as drug delivery tool instead of liposomes are being investigated (69), such as curcumin-loaded exosomes have effectively been used to target activated myeloid cells *in vivo*. During tumor growth study, dysfunctioning of immune system is needed to be appropriately explored. There is considerable role of $CD4^+$ and $CD8^+$ cytotoxic T-lympocytes in destroying antigen-specific tumor, thus the inhibition of T-cell function results in tumor growth through the suppression of immune system. Curcumin has been revealed to suppress T-cells inhibition, thus tumor cell destruction accomplishes through two processes: (i) improving the performance of effector T-

cells, and (ii) down-regulating the development of IL-10 and TGF-β in T-cells (70).

Ohno et al. (71) proposed the therapeutic use of intravenously delivered microRNA (let-7a)-loaded exosomes to target epidermal growth factor receptor (EGFR)-expressing xenograft breast cancer tissue in $RAG2^{-/-}$ mice by modifying the donor cells for the expression of transmembrane domain of platelet-derived growth factor receptor coupled with the GE11 peptide (Fig. 5).

Sun et al. reported that exosomes deliver curcumin to blood in comparatively stable form as well as in high concentration (72). They reported the increase in anti-inflammatory activity, bioavailability, stability and solubility of curcumin loaded with exosomes. The investigators treated RAW 264.7 cells with curcumin or exosomal curcumin 20 μmol/L for one hour for *in vitro* evaluation of anti-inflammatory activity of curcumin followed by the stimulation of treated cells with lipopolysaccharide (50 ng/mL) for further 6 h. After additional six hours, measurement of cytokine production (Fig. 6) in supernatant layer showed that exosomal curcumin-treated macrophages generated considerably fewer IL-6 and TNF-α as compared to curcumin alone.

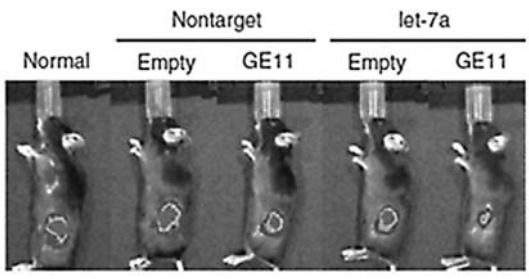

Figure 5. Figure illustrating the exosomal treatment which inhibits the growth of breast cancer after four weeks of injection (71)

Single-stranded oligonucleotides such as microRNA (miRNA) and RNA interference (siRNA) are promisingly delivered to target site *via* exosomes (84), particularly for the suppression of cancer cell growth (74). For example, RNAi and siRNA have systemically been delivered to brain through exosomes derived from dendritic cell (75).

The modulation of neovascularization has been carried out using exosomes, such as obstructing the neovascularization can obstruct the tumor growth and activating the neovascularization can activate the wound healing (76). Angiogenic function of human $CD34^{+}$ cell-derived exosomes has been studied in isolated endothelial cells (77) having proteins e.g., matrix metalloproteinases which are involved in angiogenesis (70).

A recent research found that urine-derived stem cells exosomes may have the potential to prevent kidney injury from diabetes by inhibiting

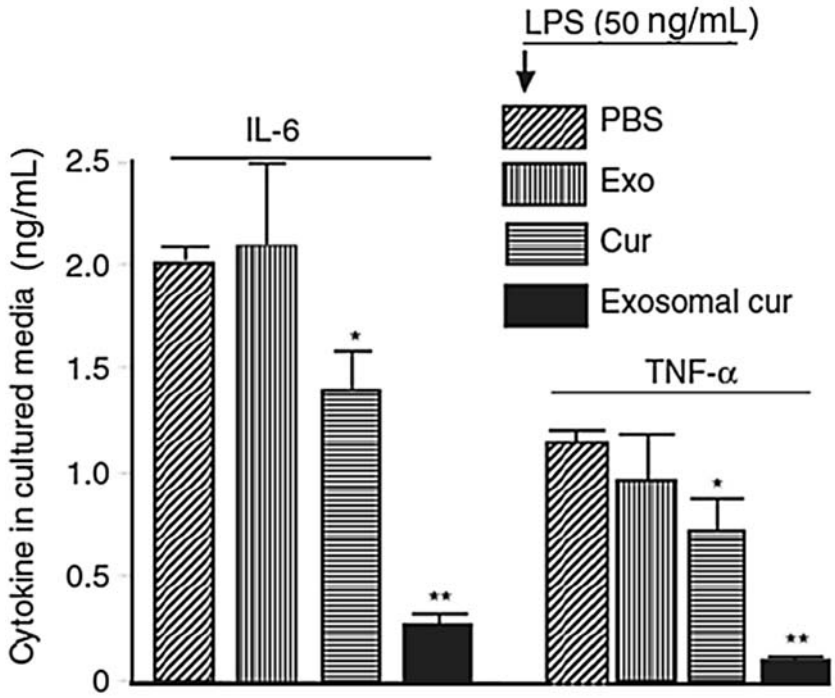

Figure 6. Diagrammatic illustration showing higher *in vitro* inhibitory effect of encapsulated curcumin on the secretion of IL-6 and TNF-α as compared to free curcumin (*$p < 0.05$, **$p < 0.01$; PBS = Phosphate-buffered saline) (72)

podocyte apoptosis and promoting vascular regeneration and cell survival (78).

CONCLUSIONS

Exosomes have emerged as a potentially safe tool for delivering drugs and RNAs due to its excellent biological features, however further studies are needed to explore and translate (from experimental animal to human) the safe and effective mode of use of exosomes for remedial applications in human.

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