Abstract

In the current study, we synthesized Copper Oxide Nanoparticles (CuONPs) by chemical and green synthesis methods. Several chemicals were used in the chemically synthesized method, whereas in green method, water extract of *Azadirachta indica (A. indica)* leaves were used. In case of both synthesis methods, Copper sulphate salt was used as a precursor. After completion of synthesis, several physio-chemical techniques were used such as FT-IR, DLS, Surface Zeta potential, EDX, XRD, SEM and TEM to confirm the synthesis of both nanoparticles. The GC mass analysis was performed to know the bioactive components of water extracts of *A. indica* leaves. The anti-oxidant property and radical scavenging property have been estimated by taking ascorbic acid as a reference. The green synthesized CuONPs showed significant anti-oxidant and radical scavenging activity, which may be due to bioactive components of *A. indica* leaf extracts. Then the toxicity difference between the chemical CuONPs and green CuONPs was evaluated in *in vitro* and *in vivo* models. The result showed that green CuONPs is less toxic compared to the chemical one. However green CuONPs showed toxicity at higher doses. Hence from the toxicity point of view, we selected green CuONPs for further study.

The *in vitro* and *in vivo* anticancer efficacy was evaluated using green synthesized CuONPs. Breast cancer (MCF-7 cells) and cervical cancer (HeLa cells) were used throughout the study and 4T1 cells were injected in subcutaneous abdominal mammary pad in Balb/c mice for solid tumor model. Green CuONPs liberate Cu ions by leaching, which internalized inside the cancer cells in a dose dependent manner. The liberated Cu ions were able to produce ROS which will indirectly induce pro-inflammatory cytokines level and then apoptosis by mitochondrial and non-mitochondrial mediated pathway.

To reduce the toxicity of green CuONPs, its surface was coated with cationic biopolymer Chitosan (CS) to reduce the toxicity and able to specifically target the cancer cells due to cationic surface charge of CS as the cancer cells were negatively charged. CS has been chosen due to its pH responsive nature that facilitates the release of Cu ions from CuONPs@CS in acidic cancer cell environment. So, the release of Cu ions from CuONPs@CS in normal cells became reduced. Surface coated green CuONPs (CuONPs@CS) showed significant apoptosis in cancer cells. The underlying mechanism of apoptosis was investigated here. The surface coated NPs induced pro-

inflammatory cytokines level and simultaneously reduced the level of the anti-inflammatory cytokines. Immuno-histochemistry and cytokine analysis showed that the involvement of cascade of Caspases is the key phenomenon of apoptosis. CuONPs@CS showed a significant reduction of tumor weight after 30 days of treatment in mice model.

The CuONPs@CS being an immunostimulant was able to activate the cellular as well as humoral immune response. The CD4+ expression and secretion of pro-inflammatory cytokines indicated the activation of T cells and macrophages which destroyed the cancer cells in *in vitro* and *in vivo* models. Alongside humoral immunity, response was triggered by the CuONPs@CS through IgG response, which indicated the adjuvant role of the nano conjugate. Th1 (Type 1 and Type 2 helper T cells) and Th2 cells were activated after the treatment with nano conjugate and acts as an immunostimulant which would inhibit the proliferation of breast cancer (MCF-7) and cervical cancer (HeLa) cells in *in vitro* and *in vivo* Balb/c mice model. The secretion of pro-inflammatory cytokines and the increase in CD4+ populations indicated the activation of immune cells in the current study. Immunotherapy by the help of metal nano conjugate (CuONPs@CS) can be an effective tool to wipe out the cancer cells from the system.

The CuONPs@CS was finally coated with folic acid to specifically target the cancer cells as the folate receptor was overexpressed on cancer cells. Green synthesized CuONPs have widespread anti-cancer activity, but the incompetent targeting ability of these metal based nanoparticles is the main obstacle. To overcome this difficulty here we synthesized a folic acid (FA) and chitosan (CS) coated nano vehicle to effectively diminish off-target effects by increasing intracellular NPs concentration in cancer cells and subsequently reducing the burden of cytotoxicity against normal lymphocytes. HeLa and MCF-7 cancer cells were used here as *in vitro* model, where CuONPs@CS@FA internalized through folate receptor mediated endocytosis pathway. Sustained release of NPs inside the cancer cells destroyed the mitochondrial membrane potential and produced ROS which caused apoptosis. 4T1 cells induced solid tumor burden in Balb/c mice, significantly reduced by CuONPs@CS@FA. Our multi layered coated conjugate could be an effective NPs delivery system for anti-cancer therapy, without creating any severe toxicity towards normal cells.

The overall study complies with the dynamic functional properties of surface coated CuONPs, which acts as a smart tool to destroy the cancer cells without significant toxicity. The nano

conjugate acts as a targeted NPs delivery system and immunostimulant, which can activate the immune cells of our system.

Keywords: MCF-7, HeLa, Cytokines, apoptotic markers, Balb/c, Copper Oxide nanoparticles, Reactive Oxygen Species, toxicity.