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Engineering Transferrin and Epirubicin onto Magnetic Nanoparticles for Targeting Drug Delivery Through Transferrin Receptors

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ABSTRACT

Cancer is a serious health problem worldwide. New nanomedicines are being developed for targeting drug delivery through receptor endocytosis process of the cell. The expression of transferrin receptors is increased in cancer cells. Transferrin and epirubicin were attached with magnetic nanoparticles (MNPs) to deliver anti-cancer drug at target site (*in vitro*). MagLISA was used to confirm the bindings of transferrin and epirubicin with MNPs by using anti-transferrin and anti-epirubicin antibodies. The MNP-transferrin-epirubicin conjugates are stable in water and carbonate buffer. 10% to 11% drug was released at pH 7.4 and in human serum but 37% to 38% drug released was observed at pH 5.5. After 96 hours, comparatively more killing effect was observed on HeLa cells (14.7%) than on normal B cells (9.8%). The binding of conjugates with HeLa cells and B cells was observed 56.83% and 39.33% respectively. The binding was confirmed (*in situ*) by immunofluorescence and immunoperoxidase microscopy using anti-epirubicin and anti-transferrin antibodies respectively. HeLa cells and B-cells treated with anti-HeLa cells and anti-B-cells antibodies were devoid of peroxidase reaction product which indicated that the receptors were masked with antibodies. Transferrin-MNPs conjugates may be considered good for targeting drug delivery.

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Authors' Contributions
ZQS conceived and designed the study. SA executed the experimental work. Both authors analyzed the data and wrote the article.

Key words
Epirubicin, magnetic nanoparticles, antibodies, microscopy, transferrin receptor

INTRODUCTION

Nanotechnology has regenerated the extensive applications of nanomaterials such as nanoparticles, nanofilms and nanowires in various subdivisions of natural sciences (Wagner *et al*., 2006; Osaka *et al*., 2008). It has opened the new era of progress in the treatment of different diseases by biodistribution of pharmaceuticals and biopharmaceuticals at target sites (Jong and Borm, 2008). Nanomaterials have proved itself as a good carrier for the proper delivery of water insoluble drugs, to cross epithelial and blood brain barriers, for co-administration of two or more drugs and for the specific delivery of medicine to the target site (Suh *et al*., 1990; O'Hagen and Illum, 1990; Kreuter, 2004; Roney *et al*., 2005; Sinha *et al*., 2006; Farokhzad and Langer, 2009). The targeted drug delivery through nanocarrier is superior and has advantages over other methods of treatment due to its stay at required sites and slow release of medicines. The advantages of nanotechnology can be realized because 24 therapeutic products based on this technology are in a process for clinical trials (Senye *et al.*, 1978).

Targeted drug delivery for specific disease can be achieved by blending the knowledge of ligand, its receptors and nanobiotechnology. The major obstacle in the treatment of cancer is targeted and efficient delivery of the drug (Senye *et al*., 1978). Anticancer drugs kill the cancer cells but also have many side effects that lead to nausea, diarrhea, loss of hair and loss of appetite, low blood count etc. Among nanocarriers, magnetic nanoparticles have shown a promising future to solve the above mentioned obstacles (Lubbe *et al*., 1996, 2001; Zhang *et al*., 2002; Neuberger *et al*., 2005; Torchilin, 2006; Wang *et al*., 2008). Magnetic nanoparticles have shown efficient binding with drugs directly or through spacer arm. Targeted drug delivery can also be achieved after binding of specific ligand with MNPs for specific receptor and applying external magnetic fields (Pankhurst *et al*., 2009) or by exploiting the increased iron uptake by cancer cells (Richardson, 2005; Chen and Chloupkove, 2009).

Many drugs are used in chemotherapy in addition to surgery and radiotherapy. Epirubicin is an anti-neoplastic drug and used for the treatment of different carcinomas (Young, 1989). Its mechanism of action is still under study but it is known to bind with DNA and inhibits the replication and transcription of DNA (Lollini *et al*., 1989). The activity of topoisomerase II becomes inactivated by epirubicin (Haldane *et al*., 1993; Robert and Gianni, 1993). Cellular metabolism in cancer cells is

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increased and the uptake of necessary metabolites for growth and development are also increased. These metabolites can also be used for targeting drug delivery (Allen, 1994; Locasale and Cantley, 2010). Transferrin may be considered as suitable candidate for targeting drug delivery due to its non-toxic, biodegradable, non-immunogenic properties as well as efficient uptake through transferrin receptors. The absorption of iron is unusually increased because the transferrin receptors are over expressed in cancer state (Qian *et al*., 2002). Transferrin is globular protein present in blood which binds with iron and iron bound transferrin absorbed through transferrin receptor by endocytosis process (Allen, 1994; Danielsa *et al*., 2006).

In this study, MNP-transferrin-Epi conjugates were prepared and characterized. The conjugates were successfully delivered to cancer cells (*in vitro*) through transferrin receptors and their binding was characterized by immunofluorescence and immunoperoxidase microscopy.The killing effect of conjugated medicines was also checked on normal B cells and HeLa cells.

MATERIALS AND METHODS

Transferrin protein

Transferrin protein was purified from healthy human serum as described (Walter *et al.,* 1967).

Development of antibodies against epirubicin, HeLa cells and human B cells

Polyclonal antibodies against epirubicin (Epi) were produced in mice. Epirubicin was conjugated with bovine serum albumin (Epi-BSA) and oval albumin (Epi-OVA) separately by two step glutaraldehyde method by following the procedure as described (Samra *et al*., 2013). Mice were immunized with Epi-BSA conjugates at 10 days interval for 4 weeks. Blood was drawn by cardiac puncture and serum was separated. The specific antibodies were affinity purified using Epi-OVA-sepharose4B as affinity resin. The presence of mouse anti-Epi antibodies was characterized by ELISA using Epi-OVA as coating antigen.

Polyclonal antibodies against B cells and HeLa cells were raised in rabbits. Total six injections of each B cells and HeLa cells were injected separately into different rabbits at two weeks intervals. For each injection, following method was adopted. The white buffy coat from human plasma was extracted on histopaque gradient and then B cells were separated by panning method using anti-CD21 antibodies. HeLa cells were maintained in DMEM medium as described (Samra *et al*., 2013). For each injection, 1 x 10^7 cells (B cells and HeLa cells separately) were washed with balanced salt solution (BSS) ($CaCl_2$ 0.0007%, glucose 10%, $MgCl_2$, 0.02%, KCl, 0.04% and 20 mM Tris-Cl pH 7.4) and separated them at 1000 rpm for 15 min at 25ºC. The cell pellet was resuspended in 0.5 ml of fixation buffer (2% glutaraldehyde in BSS) and kept at 4ºC for one hour. After centrifugation, the pellets were resuspended in 0.2 ml PBS separately and injected subcutaneously into separate rabbit. The presence of antibodies in sera was checked by ELISA using HeLa cells and B cells as coating cells separately. Whole blood was separated and IgGs were further purified by antibody purification kit as described (G Bio sciences, USA).

Indirect whole cell ELISA

Total, 1 x 10^5 cells of each HeLa cells and B cells (in 0.05 ml BSS) were mixed separately with 0.05 ml coating buffer (BSS with 2% glutaraldehyde) and added in microtitre plates. The plates were kept at 25ºC for one hour. After washing the wells with BSS, theremaining sites in wells were blocked with blocking buffer (5% skim milk in PBS). 0.1 ml of 1% H_2O_2 in PBS was added in each well to destroy the cellular peroxidase activity. Again after washing with PBS, the separate wells were treated with rabbit anti-HeLa cells and rabbit anti-B cells antibodies (1:500 dilutions) separately and then peroxidase conjugated goat anti-rabbit-IgG antibodies (1:5000 dilutions). The colour reaction was developed by using TMBsolution (tetramethylbenzidine in 100 mM sodium acetate buffer pH 6.0 containing 0.01% H_2O_2) as substrate.

Preparation of MNPs-Transf conjugates and characterization

Amine terminated magnetic nanoparticles (MNPs) were prepared as describe (Samra *et al*., 2013;2006). The nature of MNPs was determined as described (Samra *et al*., 2010). Transferrin was conjugated with amine terminated magnetic nanoaprticles by two step glutaraldehyde method. MNPs were sonicated in sonicator (Soniprep 150) at 30 KHz for 10 min on ice. Fine particles were separated, passed through 0.2 µm filter and used for conjugation. Briefly, 100 mg of MNPs were shaken in 50 ml of coupling buffer (0.01 M pyridine-Cl buffer, pH6.0) for 10 min. Particles were allowed to stand under magnetic field and supernatant was separated. This step was repeated three times and 5.0 ml of 5.0% glutaraldehyde was added to the wet cake of magnetic nanoparticles. The suspension was shaken at 100 rpm for 3 h at 25ºC. Glutaraldehyde activated MNPs were separated and washed twice with coupling buffer.

1.0 mg of transferrin protein was dissolved in coupling buffer (10 ml) (named as pre-coupling solution) and mixed with 50 mg glutaraldehyde activated MNPs.

The suspension was kept on shaker at 100 rpm for 24 h at 25°C. Magnetic nanoparticles were removed and the supernatant was saved (post coupling solution) for determining the binding of transferrin. The pellet was suspended in10 ml of 1.0 M glycine solution and kept for 30 min at 25°C. MNPs were removed and washed three times with 50 ml of wash buffer (0.01M Tris-Cl, pH 7.4, 0.15M NaCl). The conjugation was confirmed by MagLISA.

Conjugation of epirubicin with MNPs-Transf conjugate and characterization

MNPs-Transf conjugates were linked with Epirubicin (Epi) by carbodiimide activation method. 50 mg of MNPs-Transf conjugates were suspended in 20 mMTris-Cl, pH8.0 and mixed with 30 mg Epi and placed at 37°C for 20 min and then stored on ice for 25 min. 20 mg of 1-ethyl-3-(3-dimethyl-aminopropyl)-cabodiimide-HCl (EDAC) was added and pH 6.4 was adjusted with 1.0 M HCl. The mixture was kept in orbital shaker at 100 rpm for 6.0 h in dark. Again 10 mg of EDAC was added and reaction was continued for 15 h with constant shakings. The conjugates were separated by applying magnetic field and supernatant was saved for the determination of non-conjugated epirubicin. The conjugation was confirmed by Mag-LISA.

Magnetic nanoparticles linked immunosorbant assay

The binding of transferrin with MNPs and then with epirubicin was determined by MagLISA. Briefly, 0.1 ml of goat anti-transferrin antibodies (1:800 dilutions, Invitrogen) or 0.1 ml of mouse anti-Epr antibodies (1:200 dilution) was added in microtitre plates separately and incubated for one hour at 37°C. After washing, the wells were treated with blocking buffer (5% skim milk in TBS). The wells were washed with TBS and 0.1 ml of MNPs-Transf conjugates (20 µg) and 0.1 ml of MNPs-Transf-Epi conjugates (20 µg) was added in respective wells and kept for one hour at 37°C. Again after washing with TBS, 0.1 ml of TMB solution was added in each well and results were observed. The control assays only contained the antibodies in the wells. The standard curve was prepared by taking absorbance of different amount of conjugates (100, 200, 300, 400, 500 µg) in well followed by 0.1 ml TMB solution for 30 min. All assays were conducted in triplicate and average was used.

Stability of conjugate

The stability of conjugates was checked under different buffer conditions at 37°C as described below. 50µg of conjugates was mixed separately with 1.0 ml of each deionized water pH6.9, 10 mM phosphate buffer pH 5.5, 10 mM carbonate buffer pH 7.4, 10 mM phosphate buffer pH 7.4, and healthy human plasma pH 7.3 and kept at 37°C. Tubes were removed at pre-determined time (12 hours intervals) and conjugates were separated. The supernatant was used to measure optical density at 486 nm and released epirubicin was calculated from standard graph of epirubicin. The epirubicin released in water was used as reference control. All assays were completed under sterilized conditions and conducted in triplicate.

Targeting drug delivery (in vitro)

The binding of MNP-Transf-Epi conjugates with HeLa cell and B cells was determined. The HeLa cells and B cells (1.0×10^7 cells per ml) were cultured in serum free medium with 200 µg MNP-Transf-Epi conjugates for different time period (30 to 180 min) by following the procedure as described (Samra *et al*., 2013). The cells were separated and crushed in 20 mM Tris-Cl, pH 7.4 containing 0.1% Tween-20. The supernatant was separated and the presence of epirubicin in absorbed conjugates was detected by ELISA using anti-Epi antibodies. The amount of conjugates remaining in the cell culture medium was estimated by MagLISA for percentage binding of conjugates with cells.

Immunomicroscopy

Immunofluorescence and immunoperoxidase microscopy studies for binding of conjugates with B cells and HeLa cells were conducted as follow. B cells and HeLa cells were harvested after incubating with conjugates (as described above). The cells were suspended in 0.1 ml BSS and 0.01 ml of it was gently spreaded on albumin coated glass slides separately. 10% buffered formalin was dropped onto cells to fix them properly. After fixation, blocking was done with 5% BSA in PBS for 45 min in a humidified chamber. After washing of cells with 1x PBS carefully, primary antibodies (mouse anti-Epirubicin antibodies, 1:100 dilutions) was added and incubated for 45 min in humidified chamber. Again after washing, secondary antibodies (anti-mouse IgG FIT conjugated, 1:5000 dilutions) was added for 30 min in humidified chamber. Slides were rinsed gently with PBS and cells were covered with 80% buffered-glycerol.

Similarly, the B cells and HeLa cells were placed on slides and treated with 0.1% H_2O_2 to destroy the endogenous peroxidase activity. The slides were processed for immunoperoxidase microscopy using primary antibodies (goat anti-transferrin antibodies, 1:5000 dilutions) and then with secondary antibodies (anti-goat IgG, peroxidase conjugated, 1:5000 dilution). After rinsing, the slides were incubated with 1.0% diaminobenzidine solution with 0.1% H_2O_2 for 10 to 15 min. Again after washing with TBs, buffered glycerol

was added and covered with cover slips. The slides were observed under microscope (Olympus, BX51).

Conjugates binding inhibition assay

The B cells and HeLa cells (1.0 x 10^7 cells / ml) were harvested and incubated with anti-B cells antibodies (1.0 µg) and anti-HeLa cell antibodies (1.0 µg) separately and incubated for 1 h in humidified CO_2 incubator at 37°C. The antibody treated cells were incubated with MNP-Transf-Epi conjugates and processed for immunomicroscopy as described above. The results were observed under microscope.

Cell cytotoxicity assays

The effect of conjugates on the growth of B cells and HeLa cells was also checked. B cells and HeLa cells (1.0 x 10^7 cells / ml) were seeded separately in 24 wells tissue culture plates. The cells were mixed with conjugates (500 µg per well) and cultured for 4.0 days under the same conditions as described above. After every 24 h, cells were removed and the cell survival was checked by trypan blue dye exclusion method. All assays were conducted in triplicate and average was used for calculation. Non-treated B and HeLa cells were used as control.

RESULTS

Characterization of antibodies

The red colour conjugates were prepared with BSA and OVA. The total number of epirubicin molecules attached with BSA and OVA were approximately 18 to 25 respectively. Polyclonal antibodies against epirubicin was developed in mice and characterized by ELISA. The antibodies against HeLa cells and human B cells were developed in rabbits and characterized. A good titre of antibodies was achieved in sera.

Characterization of conjugates preparation

The conjugation of MNPs with transferrin (MNPs-Transf) and then with epirubicin (MNPs-Transf-Epr) was confirmed at each step. The binding was detected by exploiting peroxidase like activity of MNPs. The amino group of transferrin was successfully conjugated with amine terminated MNPs. Total 12 to 14 µg of transferrin protein was conjugated with 1.0 mg of MNPs (60% to 70% conjugation). The development of greenish colour in wells coated with goat anti-transferrin antibodies and then with MNPs-Transf conjugates showed the binding of transferrin protein onto MNPs. Similarly, 270 µg of epirubicin was conjugated with 1.0 mg of MNPs-Transf conjugates (45% conjugation). Again, the development of greenish colour in wells coated with mouse anti-epirubicin showed that the binding of epirubicin with MNPs-Transf conjugates was achieved successfully. The model for binding and characterization of transferrin and epirubicin is shown (Figs. 1 and 2).

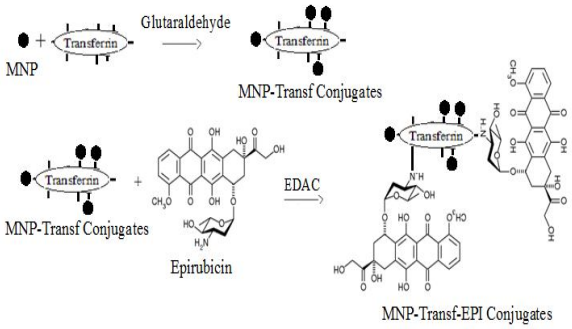

Fig. 1.A model for conjugation of MNPs with transferrin and epirubicin. The conjugation of magnetic nanoparticles with transferrin protein was completed by glutaraldehyde method. The MNPs-Tranf conjugates were further conjugated with epirubicin by carbodiimde method.

Binding of conjugates with cells

The binding of MNPs-Transf-Epr conjugates with B cells and HeLa cells was checked by MagLISA. When the assays were conducted after different time periods (half hour, one hour, one and half hour, two hours and two and a half hour), a good stable binding response was observed after 120 to 180 min. The binding response was gradually varies from 30 to 90 min. The comparative MagLISA of B cells and HeLa cells showed that the binding of conjugates was low in B cells. It was further estimated that nearly 86.32 µg (43.16%) of conjugates were present in medium of HeLa cells and 121.32 µg (60.66%) of conjugates were present in culture medium of B cells. The absorbed conjugates on B cells and HeLa cells were not estimated. On the basis of conjugates present in the medium, it was estimated that the binding of conjugates with HeLa cells and B cells was observed 56.84% and 39.34% respectively

Stability of conjugates (in vitro)

Epirubicin released profile from conjugates (*in vitro*) is shown (Fig. 3). It was determined that the drug released slowly at pH 7.4 (17%) than at pH 5.5 (34%). The drug released in plasma was found 13%. Detectable amount of epirubicin was observed after 24 h at pH 5.5 and measurable amount was estimated after 48 h at pH 5.5. The drug was not released in water and carbonate buffer.

Cytotoxic effect on cells

The cytotoxic effect of MNPs-Transf-Epr conjugates was observed by counting the total number of

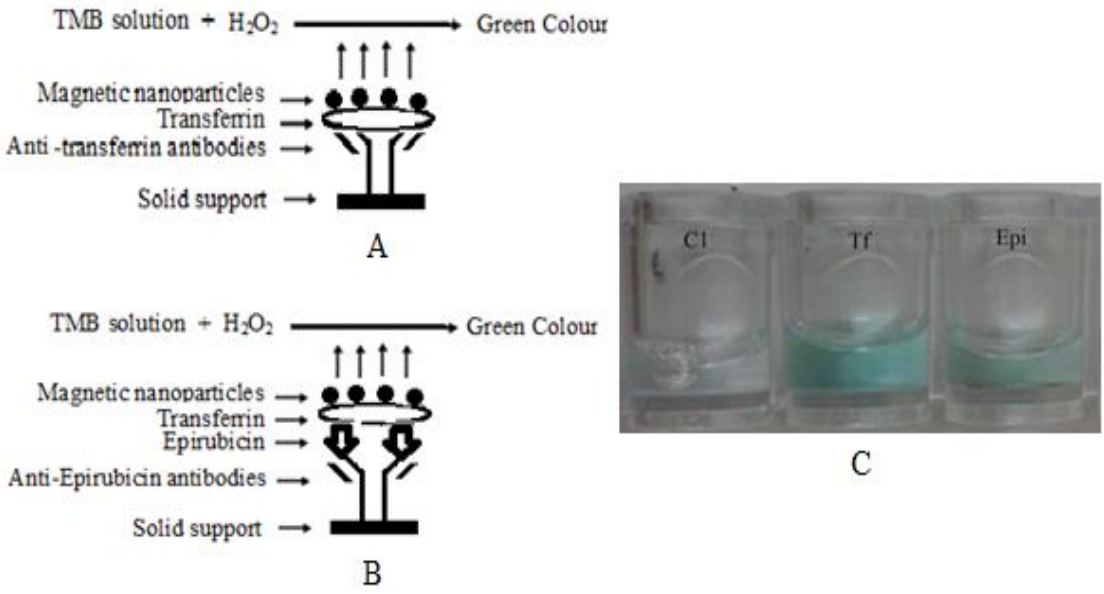

Fig. 2.Confirmation of binding of MNPs with transferrin and epirubicin by MagLISA. A) Anti-transferrin antibodies coated wells incubated with MNP-tranf conjugates. The development of greenish colour due to peroxidase like activity of MNP indicated the binding of transferrin with MNPs. B) Anti-epirubicin antibodies coated wells incubated with MNP-tranf-epi conjugates. C) The development of greenish colour due to peroxidase like activity of MNP further indicated the binding of epirubicin with MNP-epi conjugates. Control well was coated only with blocking agent. C1, control; Tf, transferring; Epi, Epirubicin.

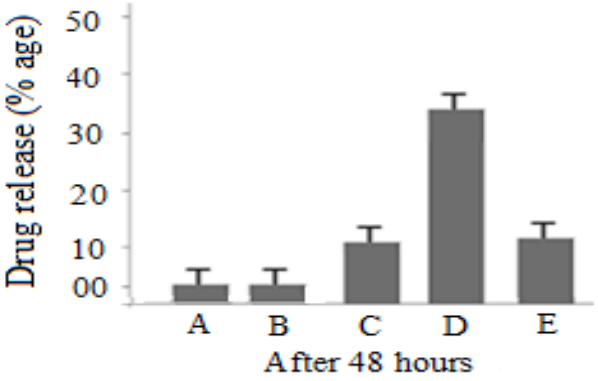

Fig. 3. Stability of conjugates. Conjugates (MNPs-Transf-Epi) were suspended in different pH buffers. A measurable amount of released drug (37 to 38%) was observed in phosphate buffer, pH 5.5 after 48 hours at 37ºC. A, deionized water pH 6.9. B, 10 mM carbonate buffer pH 7.4. C, 10 mM phosphate buffer pH 7.4. D, 10 mM phosphate buffer pH 5.5 (E), healthy human plasma pH 7.3.

survival cells at 24 h intervals (Fig. 4). After 96 h, more cytotoxic effect was noted on HeLa cells (14.7%) than B cells (9.8%). It indicated that more inhibitory effect on HeLa cells is due to better absorption of conjugates.

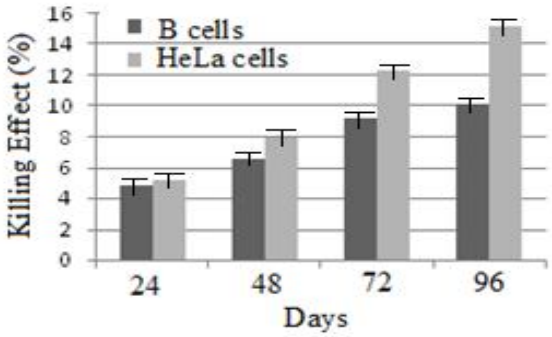

Fig. 4. Cytotoxic effect of conjugates on cell survival. HeLa cells and B cells were incubated with conjugates separately and the number of survived cells was counted by trypan blue dye exclusion test. A comparative prominent inhibitory effect (14% to 14.5%) was observed on HeLa cells. It indicated more absorption of conjugates with HeLa cells.

Immunohistochemical microscopy

The binding of conjugates with B cells and HeLa cells was also confirmed by immunofluorescence microscopy using anti-Epr antibodies and also by immunoperoxidase microscopy using anti-transferrin antibodies (Fig. 5). Comparatively, low signals of green

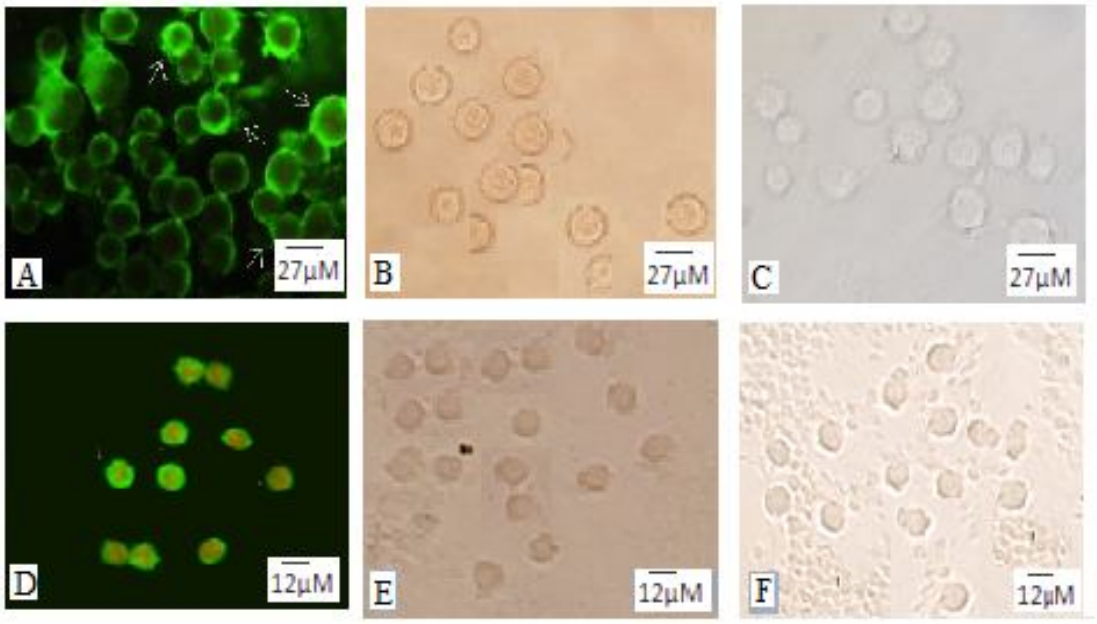

Fig. 5.Immunohistochemical labeling of HeLa cells and B cells. (Colour figure is available on line). All images were observed at 400X. HeLa cells (A) and B cells (D) were treated with conjugates for 2 h. The presence of epirubicin in binding conjugates on both cells was observed by FIT-conjugated anti-epirubicin antibodies. The green fluorescence on cells confirmed the absorption of conjugates. HeLa cells (B) and B cells (E) were also treated with conjugates for 2 h. The presence of transferrin in binding conjugates on both cells was observed by peroxidase conjugated antitransferrin antibodies. The brown colour precipataes on cells confirmed the binding of transferrin with its receptors. HeLa cells (C) and B cells (F) were also treated with anti-HeLa cells and anti-B cells antibodies respectively and processed for immunoperoxidase microscopy using anti-transferrin antibodies. The absence of brown colour product indicated the masking of transferrin receptors by antibodies.

fluorescence were observed on B cells than HeLa cells. Similarly, less deposition of immunoperoxidase reaction product was observed on B cells than HeLa cells. The B cells and HeLa cells treated with anti-B cells antibodies and anti-HeLa cells antibodies respectively were devoid of peroxidase reaction product. It further indicated that the binding of conjugates through receptor is specific.

DISCUSSION

Transferrin is an iron binding protein present in blood to import the iron into the cell through receptors. It is considered as a good carrier for targeting drug delivery of anti-cancer medicines due to high expression of transferrin receptors on cancer cells. In this study, transferrin was conjugated with anticancer medicine “epirubicin’ and MNPs for targeting drug delivery to HeLa cells and normal B cells (*in vitro*). Transferrin was conjugated with MNPs by glutaraldehyde activation method and then with epirubicin by carbodiimide activation method. The conjugation of transferrin and epirubicin was confirmed by the development of greenish colour in MagLISA. It was estimated that average 60.75% transferrin protein was immobilized per 1.0 mg of MNPs and 45% of epirubicin was conjugated per 1.0 mg of MNPs-Transf conjugates.

Epirubicin was separately conjugated with BSA and OVA by glutaraldehyde method. Number of molecules of epirubicin attached with BSA and OVA were 14 to 20 respectively. Conjugates of epirubicin with BSA were used to immunize the mice and conjugates of epirubicin with OVA were used to characterize antibodies. Antibodies against HeLa cells and B cells were developed and characterized for conjugates binding inhibition assays at target sites. All immunochemical tests confirmed the successful production of antibodies.

The conjugates MNPs-Transf-Epr were used for targeting the cancer cells. Normal human B cells were isolated and used as control. For targeted drug delivery, HeLa and B cells were cultured separately with MNPs-Transf-Epr conjugates for different time periods at 37°C. It was observed that culturing of cells with conjugates for 120 min showed optimum binding of conjugates with cells. A 43.16% of conjugates were present in medium of

HeLa cells (56.84% absorption) on cells and 60.66% conjugates were present in medium of B cells (39.34% absorption) on cells. In immunomicroscopy, the presence of green fluorescence indicated the binding of epirubicin and brown precipitates indicated the presence of transferrin on the cells. The HeLa cells and B cells were also treated with anti-HeLa cells and anti-B cells antibodies for masking the surface receptors. The absence of brown precipitates indicated the specific targeting drug delivery through transferring receptors.

The epirubicin released from conjugates was observed after 12 h at pH 5.5 as well as in human plasma. The release of epirubicin was comparatively high at pH 5.5 than other pH values and in human plasma. The release of drug at low pH further advocated the hypothesis of drug release at low pH during endocytic pathways. The enzymatic hydrolysis of –N-N bond and –C-N bond at low pH in lysosomic pathways will be favorable for drug release and to block the proliferation of tumors or cancer cells. Epirubicin is used as anti-cancer drug to kill and control the rapidly dividing cancer cells. It is also observed that the anticancer drug not only absorbed by the cancer cells but also disturb the normal physiological conditions of the healthy cells. So the absorption of drug through transferrin will be beneficial for targeting drug delivery. The green fluorescence on cancer cells confirmed the successful delivery of conjugate. The B cells also produced fluorescence which is due to the presence of normal transferrin receptors but the high fluorescence was seen on cancer cells which are due to over expression of transferrin receptors. The qualitative analysis of conjugates binding with HeLa cells and B cells was checked in cell lysate by ELISA after destroying the endogenous activity of alkaline phosphatase. Comparatively more yellow color was observed in HeLa cells lysates than B cells lysates used for ELISA. It also indicated that uptake of conjugate through transferrin receptors is increased in cancer cells as compared to normal B cells.

Both transferrin and epirubicin were immobilized onto MNPs to make it suitable carrier for dual role. One was the interaction of transferrin ligand and its receptor and second is to stabilize the conjugation of transferrin and epirubicin. Iron uptake is increased in cancer cells, so, MNPs can augment the uptake of medicine through transferrin receptor endocytosis process of HeLa cells. Transferrin may be used as carrier for targeting drug delivery due to its over expression in ratio about 50,000 to 100,000 receptors on cancer cells (Inoue *et al*., 1993) and secondly it also facilitates the absorption of conjugated drug across the blood-brain barrier (BBB) (Qian *et al*., 2000). In neurodegenerative diseases, iron levels are also increased and transferrin attached to therapeutic drugs may be used for the treatment (Qian *et al*., 2002). Another reason for using transferrin as a ligand is to reduce drug resistance mechanism of cells. The resistance is due to drug resistant proteins such as p-glycoprotein (P-gp) and drug resistant behavior is generally shown by the tumour cells (Qian and Shen, 2001; Wu *et al*., 2007).

The integration of drug at target sites at cellular level is an important route to observe the efficiency of anti-cancer drugs. In order to avoid multidrug resistance in cancer cells in non-conjugated form, the drugs may be used for treating the disease after binding with suitable carrier.

Different nanoparticles (NPs) are being considered as a good carrier in nanomedicines for drug delivery through lipid-dextran NPs, dendrimers, polymeric NPs, MNPs and ceramic NPs. MNPs are considered as a promising candidate to remove many problems linked with drug delivery. Another advantage of MNPs is small in size which can absorb into cells easily and smoothly by endocytosis process. Further studies are being conducted to observe the endocytic mechanism of conjugated medicines through transferrin, glutamine and folate receptors.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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