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Article Title: **Vitro Co-Delivery of Decarbazine and Photosense loaded Exosome Nanocarrier for PDT based Combinational Therapy**

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Abstract:
Exosomes are emerging as a natural nanocarrier having a potential to replace the synthetic nanoparticles in drug delivery applications due to their low immunogenicity and high biocompatibility. The objective of present study is to use exosomes as a nanocarrier for delivery of anticancer drugs during Photodynamic therapy (PDT) alone as well as in combination with the chemotherapy (combinational therapy).
In this study, exosomes were isolated and loaded with photosense (AlPc4) and decarbazine (DTIC, 5-3,3-(dimethyl-ltriazeno) imidazole-4-carboxamide) drug alone and in combination with 25nM and 250 nM concentrations. Further, these exosomes (blank as well loaded) were used during cytotoxicity test, PDT and combinational therapy on rhabdomyosarcoma (RD) cells. Exosomes either loaded or unloaded showed no cellular toxicity (100% cell viability) on rhabdomyosarcoma (RD) cell culture, making them a biocompatible nanocarrier. On comparison, exosome mediated PDT showed more cell death alone then in combination with the chemotherapy (combinational therapy). Light inverted microscopic images showed cell swelling, and membrane rupture indicating the presence of necrosis during PDT and combinational therapy mediated cell death. Furthermore, expression analysis was carried out of five apoptosis related genes (CASP3, CASP9, P53, FOXO3a and PARP1) which shows downregulation in all treatment arms (cytotoxicity, PDT and combinational therapy).
On the basis of the results obtained, the absence of apoptotic mechanism is indicated during both PDT and combinational therapy in RD cells.
Keywords: Exosomes, Rhabdomyosarcoma, Photodynamic therapy, Combinational therapy.

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1. Introduction

From a past decade, the nanoparticles (NPs) have been extensively studied in medicinal field during treatment, diagnosis and drug delivery in a very attractive way. Nano based drug delivery systems have emerged as a safer and promising platform in cancer treatment by showing better pharmacokinetics, less drug resistance and side effects [1-3]. NPs basically help in improved therapeutic efficacy due to better loading and releasing factors [4,5], circulation-time of drug and biocompatibility [6-8] and in final step target-specific and controlled delivery of drugs [9-12]. NPs have also shown increased permeability and retention effect (EPR) [13,14]. But there are certain limitations in use of nanoparticles as drug delivery system for cancer treatment such as presence of targeting ligand might lead towards increased immune-elimination [15] and might also not be specific for different tumors, as receptors can be different due to phenotypic heterogeneity or versatile genetics of tumors [16,17].

To overcome these limitations exosomes have emerged as attractive nanocarriers for cancer-drug delivery and alternative to NPs [18] due to unique physicochemical and structural features like biocompatibility, stability in circulation, low toxicity and low immunogenicity [19-21].

Exosomes are subgroups of extracellular vesicles with size range of 40-150 nanometer in diameter size, having lipid bilayer membrane [22, 23]. In 1980s, exosomes were identified in extracellular space [24]. These molecules are functional vehicles which carry cargo of nucleic acids [25-27], lipids [28] and proteins [25]. Exosomes deliver cell specific cargo material and alter phenotype and behavior of target cell, so function as mediators of intercellular communication [29]. Exosomes play role in several complex biological pathways and diseases which also support their application in diagnostic and therapeutic vehicles in immune response pathways [30-32], infectious agents [33,34], antigen presentation [35], signal transduction [36] and different cancers [23,37]. Moreover, tumor cells produce increased number of exosomes than normal healthy cells which might support tumor proliferation through altering immune system, neighboring parenchymal tissue and angiogenesis [38,39].

In this study, we have used rhabdomyosarcoma (RD) which is adolescent muscle cancer that accounted for 50% soft tissue sarcomas in children and 7-9% of pediatric tumors [40, 41]. RD originated from skeletal muscle precursors [42] and categorized by myoblast-like undifferentiated cells [43]. RD can also occur in voluntary muscles of head and neck, trunk, and limbs [44]. Different therapeutic approaches have been used to treat RD including irradiation,

chemotherapy, and surgery. During these treatments, the healthy cells of the cancer patient body also get effected [45]. So targeted and effective treatment strategy with less side effects is urgently required for treatment of cancer [46-50].

Among the different cancer therapeutic options available, photodynamic therapy (PDT) has been introduced as more accurate, selective and less-invasive strategy having a potential to target the cancer cells [51-54]. PDT combines photosensitizing drug, photons of light with specific wavelength and tissue oxygen that produce cytotoxic compounds of short-lived oxygen radicals [55, 56]. Photosense (AlPc4) is a phthalocyanine photosensitizer [57, 58], which has been approved for PDT in cervical cancer [59-61], bladder cancer [62], choroidal tumor [63] and sarcoma [64]. Furthermore, studies have reported that PDT in combination of chemotherapy results in either synergistic or antagonistic effect depending on photo chemo drug interactions. [65-67]. (DTIC, 5-3,3-(dimethyl-l-triazeno) imidazole-4-carboxamide) is commonly termed as dacarbazine which is a chemotherapeutic drug that possesses a potential role for producing methyl diazonium cation in liver behaving as a major factor for anti-cancer metastasis [68].

2. Methodology

2.1. Isolation of Exosomes

Ultracentrifugation method was used for isolation of exosomes. For this, 18.5 ml of phosphate buffer solution (PBS) was added in 0.75 ml of fresh blood (from healthy individual) and the resulted blood solution was centrifuged at 2000xg for 10 minutes at 4°C. The obtained supernatant was separated and again centrifuged at 20,000xg for 40 minutes at 4°C. The resulting supernatant was filtered using 0.22 um filter and the centrifuged at 118,000xg for 70 minutes at 4°C. After this, the supernatant was discarded and the obtained pellet was properly washed and stored in 20ml PBS at -20°C.

2.2. Drug Loading and Characterization of Exosomes

Isolated exosomes were loaded with a chemotherapeutic drug (dacarbazine) and a photosensitizer (photosense). For this, the nano drug solution was added to the exosome solution with 1:1 ratio and incubation was performed for 30 minutes at room temperature. The solution obtained was centrifuged at 118,000xg at 4°C for 70 minutes. The pellet obtained was washed with PBS and stored at -20°C.

Dynamic light scattering (DLS) was performed to characterize/analyze the exosome nanoparticles by calculating their hydrodynamic size and polydispersity index. Further, fluorescence spectroscopy was performed to observe the loading efficiency of the photosense (25 nM and 250 nM) and decarbazine (25 nM and 250 nM) loaded on exosome nanoparticles.

2.3. Cell Culturing and Calorimetric assay

Culture of Rhabdomyosarcoma (RD) cell line was obtained from National Institute of Health (NIH), Islamabad and properly maintained using 5 % minimum essential medium (MEM) on 96 well plate. Light inverted microscope was used to observe the cells at different steps. The cell culturing plates were prepared in duplicates with same labelling and treatment to use further for MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and expressional analysis.

The RD cells were allowed to incubate with the exosome nanoparticles (blank as well as loaded) with known dilutions in triplicates for 24 hours. Following this, the photodynamic therapy was performed by radiating two different energies (2 joule/cm^2 and 5 joule/cm^2) on cell cultured wells containing photosense and photosense cum decarbazine loaded exosomes and again incubation was performed for 24 hours. Further, the MTT assay was performed.

2.4. Combinational Index

During combinational therapy, the synergistic and antagonistic effect of photosense and dacarbazine was determined by calculating the combinational index (CI) of both drugs through Chou-Talalay method.

2.5. RNA expression and RT-PCR

After 24 hours of incubation, the calorimetric assay plates were taken out and cells were treated with the lysis buffer. Cell lysate was isolated and Trizol reagent was used for RNA extraction**.** Further, the Revert Aid 1st strand cDNA synthesis kit was used to synthesize the complimentary cDNA from the obtained RNA. Further, the relative expression of *CASP3*, *CASP9*, *P53*, *PARP1* and *FOXO3a* was analyzed through Quantitative real time PCR.

2.6. Statistical Analysis

Origin and Graph pad prism software were used to analyze the results obtained. “Student t-test” and “one way of variance ANOVA was used to evaluate the association between expression level of *CASP3*, *CASP9, P53, FOXO3a* and *PARP1* and different treatments. The statistically significant value was adjusted at $p \leq 0.05$.

3. Results

3.1. Dynamic light scattering (DLS)

The exosomes isolated were characterized through DLS by Microtrac Nanotrac Wave-II, (USA). The average size of blank exosomes was observed as 59.03 nm whereas, the obtained

polydispersity index (PDI) and zeta potential value was 0.212 and 34.5 mV as shown in Figure 1.

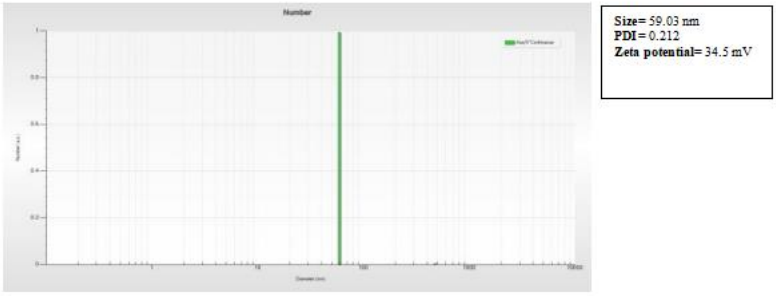

Figure 1. Dynamic light scattering mean size, polydispersity index and zeta potential of isolated exosomes.

3.2. Fluorescence Spectroscopy

The Fluorescence spectroscopy (Flouromax-4, Horiba Scientific Jobin Van, USA) was used to analyze the loading efficiency of drugs. For this, two different drugs i-e decarbazine and photosense with two different concentrations (25 nM and 250 nM each) were used and 6 different samples were prepared. Two samples were prepared as decarbazine loaded nanoparticles (one with 25 nM and the other as 250 nM drug concentration), two samples as photosense loaded nanoparticles (one with 25nM and the other as 250nM drug concentration) and two samples as decarbazine and photosense loaded nanoparticles (one with 25 nM and the other as 250 nM drug concentration).

The PBS solution of decarbazine and decarbazine loaded exosomes with 25 nM and 250 nM concentrations were excited with an excitation wavelength 330 nm. Whereas the PBS solution of photosense and photosense loaded exosomes with 25 nM and 250 nM concentration were excited with an excitation wavelength of 400 nm. The solution of decarbazine plus photosense loaded exosomes with 25 nM and 250 nM in PBS was exciting first at 330 nm and then at 400 nm wavelength.

When PBS solutions of decarbazine, decarbazine loaded exosomes and decarbazine plus photosense loaded exosomes were excited with 330 nm wavelength, then prominent and highest peaks was observed at 372 nm wavelength as shown in Figure 2(A) and 2(B).

For PBS solutions of photosense, photosense loaded exosomes and decarbazine plus photosense loaded exosomes were excited with 400 nm wavelength as a result of which different peaks were observed. The highest peaks observed were at 465 nm, 463nm and 461nm for 25 nM concentration and 460 nm, 463 nm and 435 nm for 250 nM concentration respectively as shown in Figure 2(C) and 2(D).

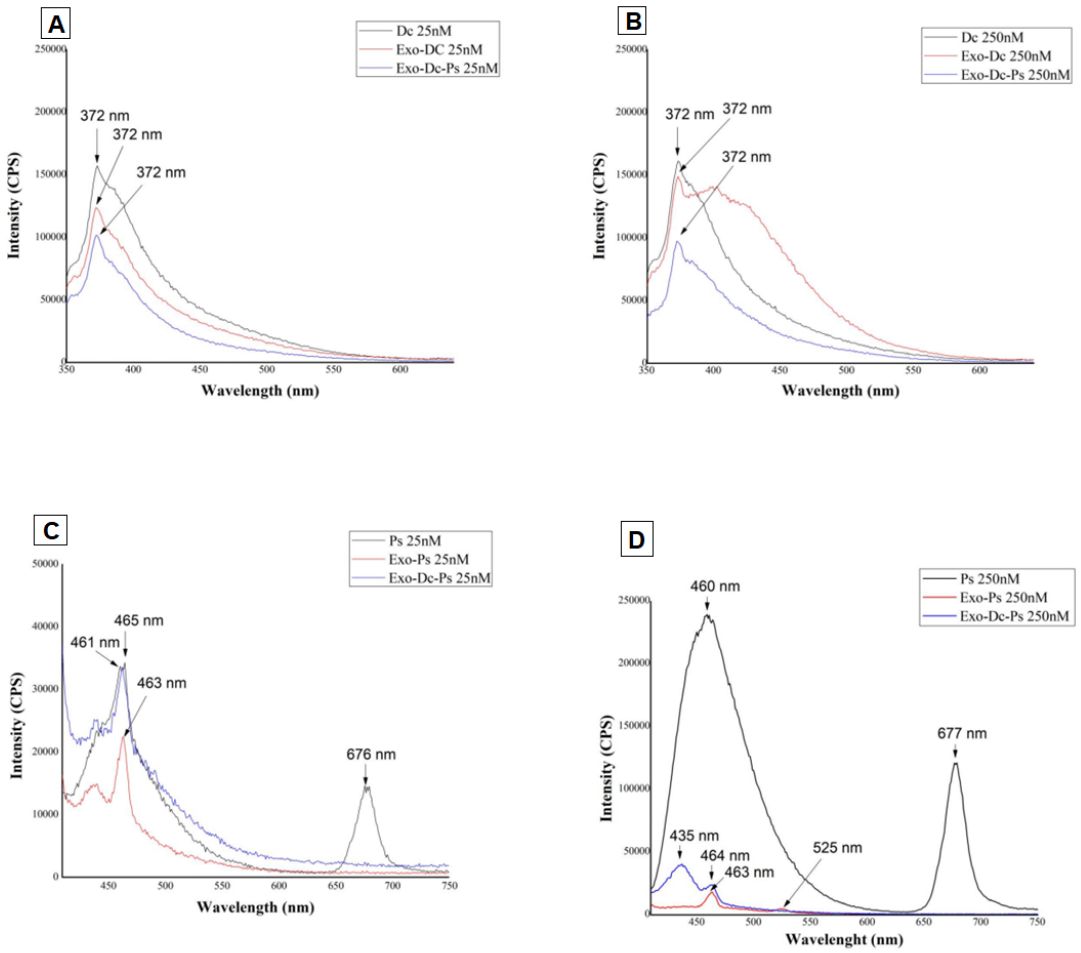

Figure 2. Fluorescence spectroscopy results of dacarbazine and photosense loaded exosomes. (a) and (b) represents the fluorescence graph at 330 nm whereas (b) and (c) represent the fluorescence graph at 400 nm respectively.

3.3. MTT Assay

In order to analyze the percentage (%) viability of exosomes (loaded or unloaded with drugs) on rhabdomyosarcoma (RD) cell line, the cytotoxicity test was performed. On the basis of results obtained in Figure 3, no cellular death was shown in all treatment arms thus clearly indicating that our isolated exosomes (either loaded or unloaded) are successfully biocompatible with the rhabdomyosarcoma cell line.

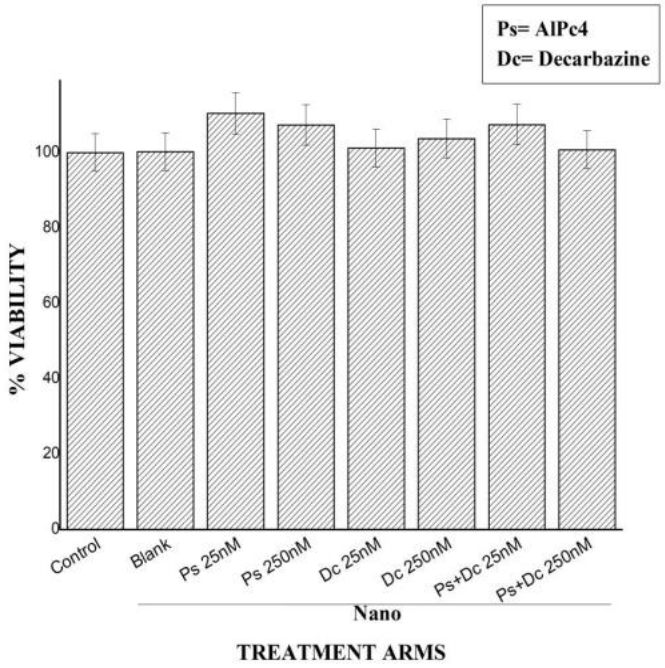

Figure 3. Evaluation of cytotoxicity of blank and loaded exosomes on RD cell line.

Exosome mediated photodynamic therapy (PDT) was performed on RD cells using photosense loaded exosomes with 25 nM and 250 nM concentration. Laser diode was used with two different energies i-e 2 J/cm^2 and 5 J/cm^2. The obtained results showed highest cell death on treatment arm of photosense (250 nM) loaded exosomes when 5 joule/cm^2 of energy of laser diode was irradiated with 30% cellular viability as shown in Figure 4A. Microscopic images during the treatment are shown for all treatment arms in Figure(B-G).

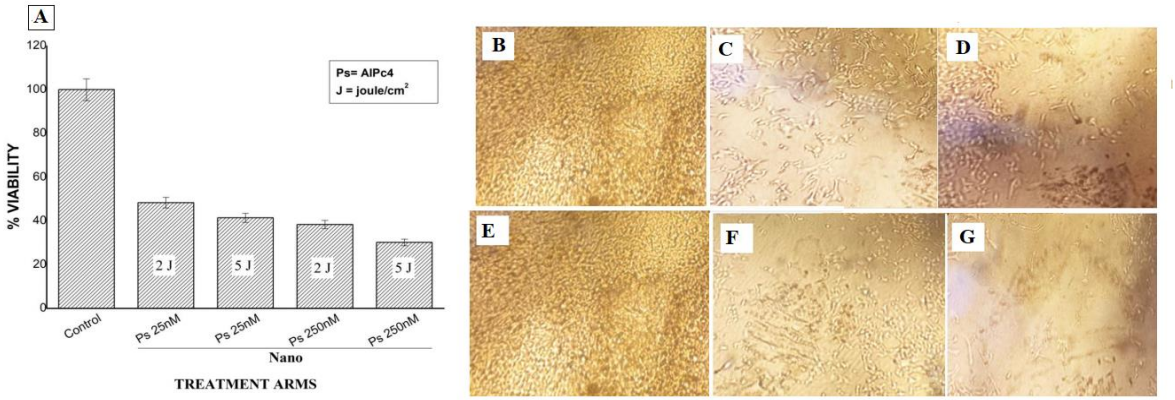

Figure 4. (a) represents the MTT assay results of photodynamic therapy with 25 nM and 250 nM photosense (AlPc4) loaded exosomes on RD cell line, (b) and (e) represents the microscopic image control cells, (c) represents the microscopic image of PS 25 nM treated cells with 2 J, (d) represents the microscopic images of PS 25 nM treated cells with 5 J, (f)

represents the microscopic images of PS 250 nM treated cells with 2 J, (g) represents the microscopic images of PS 250 nM treated cells with 5 J.

Further for combinational therapy (photodynamic therapy was combined with chemotherapy) using decarbazine and photosense loaded exosome (25 nM and 250 nM) and laser diode was irradiated using two different energies of laser diode (2 joule/cm^2 and 5 joule/cm^2). As shown in Figure 5(A), the highest cell death was shown in the treatment arm of decarbazine and photosense loaded (250 nM) using 2 joule/cm^2 energy with 51% cellular viability. The microscopic images for all treatment arms are shown in Figure 5(B-G).

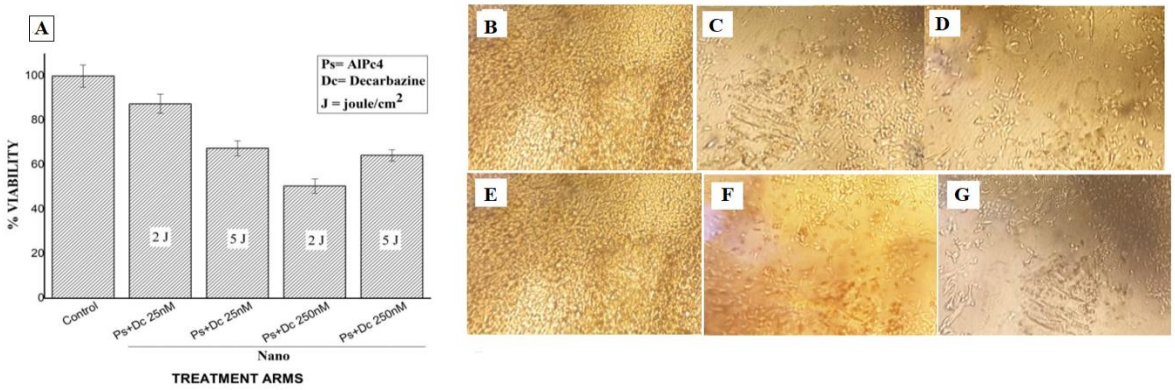

Figure 5. (a) represents the MTT assay results of combinational therapy with 25 nM and 250 nM photosense (AlPc4) and decarbazine (Dc) loaded exosomes on RD cell line, (b) and (e) represents the microscopic image control cells, (c) represents the microscopic image of PS+Dc 25 nM treated cells with 2 J, (d) represents the microscopic images of PS+Dc 25 nM treated cells with 5 J, (f) represents the microscopic images of PS+Dc 250 nM treated cells with 2 J, (g) represents the microscopic images of PS+c 250 nM treated cells with 5 J.

3.4. Combinational Index for Decarbazine and Photosense Drugs

The drug combinational effect was measured quantitatively by Chou-Talalay method in order to find out the combinational index (CI) value of decarbazine and photosense drug. The values obtained are shown in Table 1.

Table 1. Represents the combinational index (CI) values of drugs (photosense and decarbazine) with two different concentrations (25 nM and 250 nM). CI < 1 shows the synergistic effects of the drugs whereas CI= additive and CI > 1 shows the antagonistic effect. According to the calculations performed, the combinational therapy showed CI<1 for both doses of drugs i-e 25 nM and 250 nM concentrations indicating synergistic effect of dacarbazine with photosense.

S/No	Combinational arms	PDT (2 joule/c	PDT (5 joule/cm2)
1	Photosense + Decarbazi (25 nM each)	0.87	0.32
2	Photosense + Decarbazi (250 nM each)	0.22	0.68

3.5. Relative Expression for *CASP3*, *CASP9*, *P53*, *PARP1* and *FOXO3a*

Relative expressions of *CASP3*, *CASP9*, *P53*, *PARP1* and *FOXO3a* were observed in RD cells for cytotoxicity test, photodynamic therapy, and combinational therapy. Student t test was performed for analysis of results as shown in Figure 6,7 and 8.

Based on results obtained for *CASP3*, relative downregulation was observed in all treatment modalities (cytotoxicity (p=0.1346), PDT (P=0.0302) and combinational therapy (p=0.0065)) as shown in Figure 6(A, B&C)). In case of *CASP9* gene, expressional downregulation was observed in all treatment modalities (cytotoxicity(p=0.2072), PDT(p=0.6911), combinational therapy(p=0.0122)) as shown in Figure 6(D, E&F).

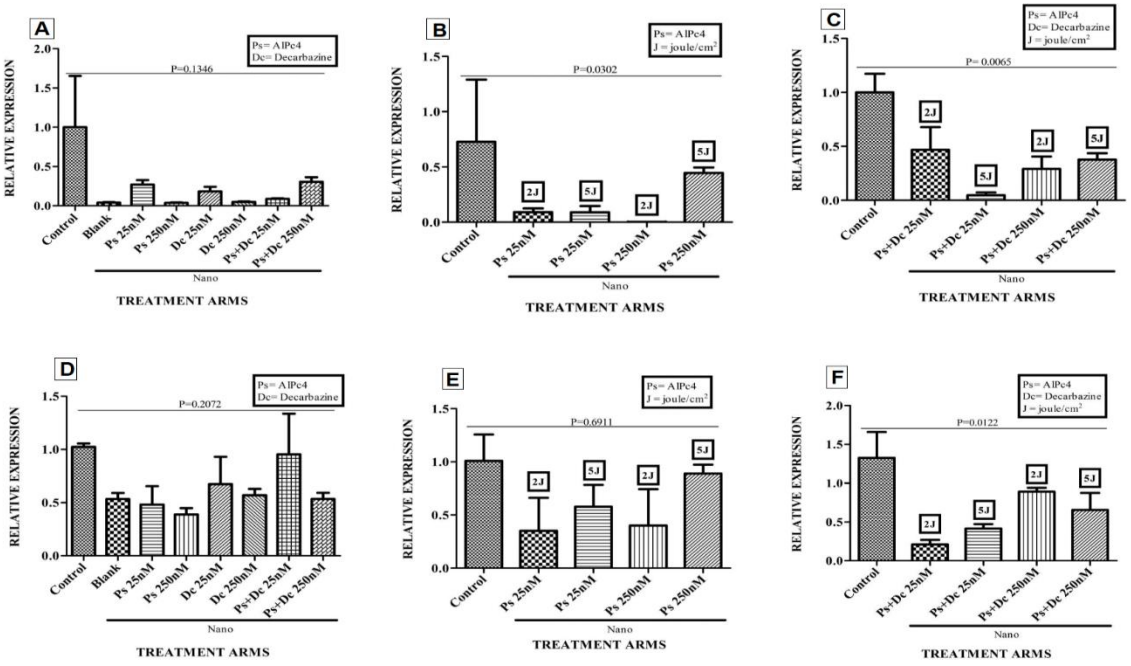

Figure 6. Relative expression of *CASP3* and *CASP9* during (A) and (D) cytotoxicity test of blank and loaded exosomes on RD cells (B) and (E) photodynamic therapy on RD cells by using photosense loaded exosomes, (C) and (F) combinational therapy on RD cells by using decarbazine and photosense loaded exosomes.

P53 gene downregulation was again observed in all treatment arms (cytotoxicity(p=0.9321), PDT(p=0.005) and combinational therapy(p=0.059)) as shown in Figure 7(A, B & C) respectively. Relative expressions of *PARP1* were also downregulated in all treatment assays

(cytotoxicity (p<0.001), PDT(p=0.2234) and combinational therapy(p=0.433)) as shown in Figure 7(D, E&F).

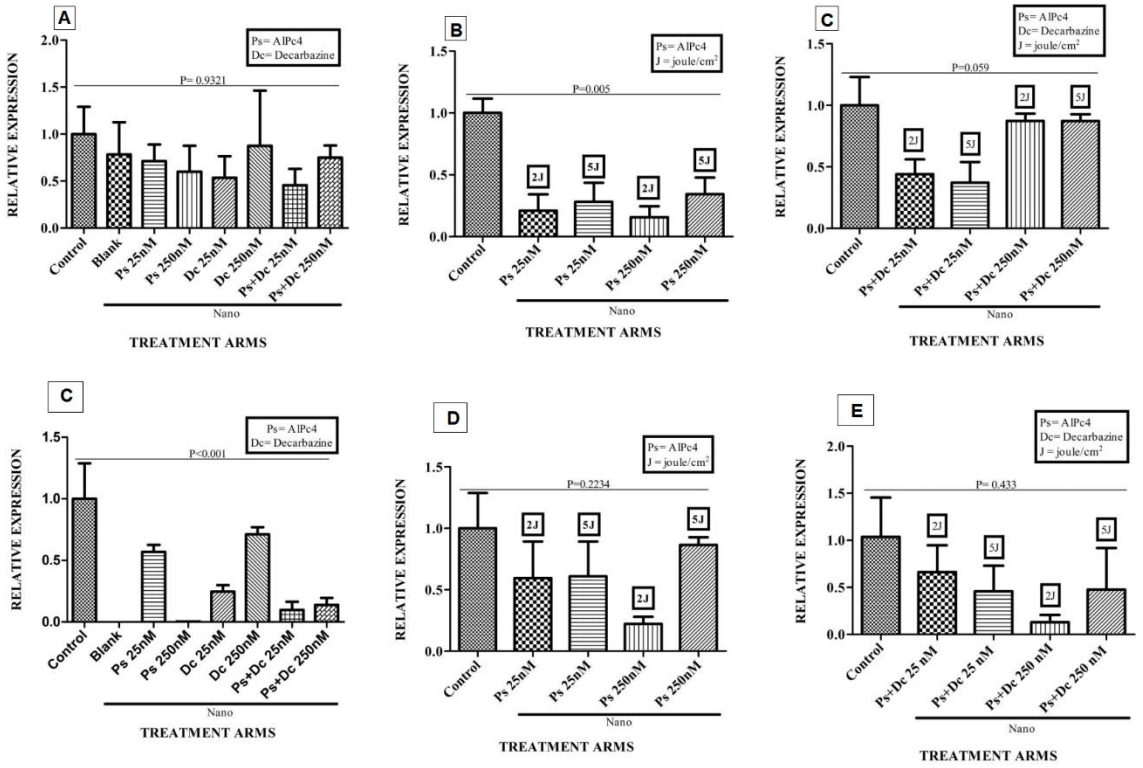

Figure 7. Relative expression of *P53* and *PARP1* during (A) and (D) cytotoxicity test of blank and loaded exosomes on RD cells (B) and (E) photodynamic therapy on RD cells by using photosense loaded exosomes, (C) and (F) combinational therapy on RD cell by using decarbazine and photosense loaded exosomes respectively.

For *FOXO3a* gene, again downregulation of relative expression was analyzed in all treatment assays (cytotoxicity(p=0.2681), PDT(p=0.0415), combinational therapy(p=0.0098)) as shown in Figure 8(A, B&C) respectively.

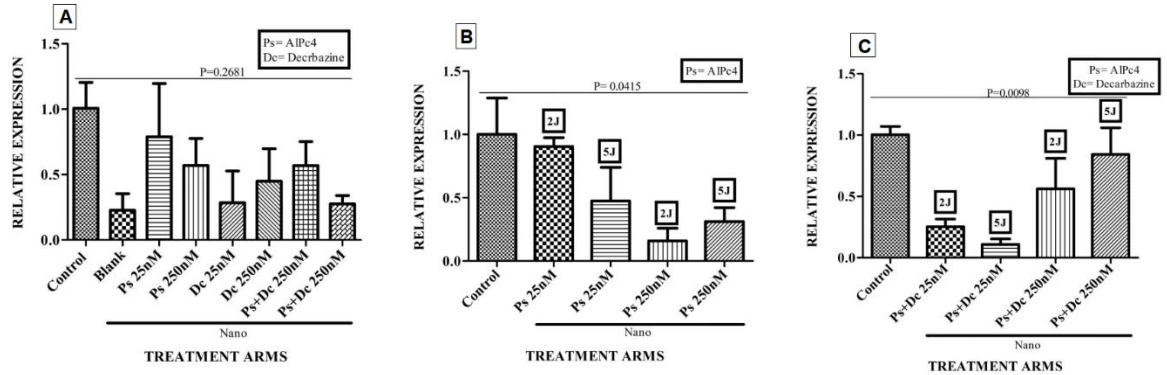

Figure 8. Relative expression of FOXO3a during (A) cytotoxicity test of blank and loaded exosomes on RD cells (B) photodynamic therapy on RD cells by using photosense loaded

exosomes, (C) combinational therapy on RD cell by using decarbazine and photosense loaded exosomes.

4. Discussion

Due to minimal immunogenicity, good biocompatibility and potential to cross cell membrane, the exosomes are emerging as a promising biological nanocarrier for drug delivery applications. [69-73]. Still a lot of research is needed to make exosomes an excellent nanocarrier to target different cells and tissues for cancer therapy [74,75]. In the present study, we have isolated exosomes from blood by using ultracentrifugation method [76]. The isolated exosomes were then loaded with dacarbazine and photosense alone as well as in combination with 25 nM and 250 nM concentration.

According to the results obtained from dynamic light scattering, the mean particle size of isolated exosomes was 59.03 nM which is within an excellent range [76,77]. The polydispersity index (PDI) value was 0.212 which is in accordance with Gomes *et al*., (2022). Further, the value of zeta potential obtained was 34.5mV which strongly suggests monodispersity within the sample [78,79]. All these obtained results (mean particle size, PDI and zeta potential) make the isolated exosomes as an acceptable carrier for nano drug delivery [80-82].

Following this, the loading of both drugs i-e dacarbazine (chemotherapeutic drug) and photosense (photosentisizing drug) was confirmed with the help of fluorescence spectroscopy [82-84]. Thus, the results obtained strongly confirmed that dacarbazine was more efficiently loaded on exosomes as compared to the photosense [85].

After this, cytotoxicity test for exosomes (blank and loaded) was performed on rhabdomyosarcoma (RD) cell line and because of this, no significant cell death was observed making these nanocarrier biocompatible in order to use them in drug delivery applications [86-88].

Following this, PDT was performed using photosense (25 nM and 250 nM) loaded with exosomes on RD cells. During this, laser diode with two different energies i-e 2 J/cm^2 and 5 J/cm^2 was applied. Results observed were encouraging showing significant cell death with the highest cell death of 70% for photosense (250 nM) loaded exosome with irradiation of 5 joule/cm^2 energy of laser diode. These results are in accordance with the previously reported study by Jang *et al*., (2021). The results obtained indicate that the cell death of rhabdomyosarcoma cells was significantly increased by increasing the energy of laser diode [68, 91-93].

Further, combinational therapy was performed using photosense and dacarbazine (25 nM and 250 nM) on rhabdomyosarcoma (RD) cells. For 25 nM concentration, an increase in energy from 2 J/cm^2 to 5 J/cm^2 showed more cell death which is in accordance with the reported data by Arshad *et al*., (2022) and Miyamoto et al., (2011). Whereas, for 250 nM concentration, an increase in energy of laser diode from 2 J/cm^2 to 5 J/cm^2 showed less death of RD cells as previously reported by Al-Qatati *et al.,* (2017).

Combinational index of both drugs i-e photosense and decarbazine was calculated during combinational therapy. Both doses (25 nM and 250 nM) of photosense and dacarbazine showed synergistic effect but unlike 25 nM concentration, the 250 nM concentration drugs showed an increase in CI by increasing the energy of laser diode from 2J/cm^2 to 5 J/cm^2. This strongly suggests that after increase of the intracellular cumulative dose from the threshold value of drug, the synergistic value may reverse as compared to the lower dose end [65, 68]. Considering different signaling pathways of activation or in-activation, further research may be carried out to observe different combinational doses response in that narrow range.

Cell swelling and cell membrane disruption [95,96]. were morphologically observed in the microscopic images of RD cells during PDT and combinational therapy as shown in Figs. 4 & 5 (B, C, G, E, F) strongly indicating the happening of necrosis [97-100].

Relative expression of five apoptosis related genes i-e *CASP3*, *CASP9*, *P53*, *PARP1* and *FOXO3a* was analyzed by quantitative real time polymerase chain reaction on cells of rhabdomyosarcoma.

During the process of apoptosis, *CASP3* and *CASP9* gets activated by cytochrome c through activation of mitochondrial pathway. Both genes were down regulated, indicating low transcription, protein expression and activation of enzymes in RD cells during all three tests (cytotoxicity, PDT and combinational therapy).

P53 is a tumor suppressor gene which binds with different death receptors and pro-apoptotic genes like BCL-2 family. According to the previously reported literature, the expression of *P53* gene is decreased with an increase in *MDM2* gene in RD cells which plays a central role in inhibition of apoptotic process [101,102].

On the basis of regulation and functional, P53 and PARP1 exhibit significant similarity. Both proteins target similar proteins to prevent the apoptotic mechanism by using different mechanisms [106]. FOXO3a was downregulated which ultimately decreased the production of key apoptotic proteins i-e PUMA and BH-3Only that lowers the apoptotic mechanism in RD cells [106-107].

PARP1 protein acts as a substrate for caspases and its production starts after the initiation of apoptotic process. This protein is cleaved by *CASP 3* and *CASP7* [105] which is considered as a hallmark of apoptotic process [106,107]. So, downregulation of *PARP1* results in decrease of apoptotic process within RD cells [108]. Downregulation was observed in all five (*CASP3*, *CASP9*, *P53*, *PARP1* and *FOXO3a*) genes clearly indicate that apoptosis mechanism was absent in RD cells while performing PDT and combinational therapy [109].

5. Conclusion

In present study, isolated exosomes (blank as well as loaded) showed good biocompatibility with RD cell line. Exosome based combinational therapy of PDT and chemotherapy showed antagonistic effect between dacarbazine and photosense drug. Further, downregulation of apoptosis related genes indicates the absence of apoptotic mechanism in RD cells. The cell death mode involved during PDT and combinational therapy may be necrosis. Further, more research is needed to observe the combinational dose behavior of decarbazine and photosense drug in different ranges of concentration and analyzing different signaling pathways activation or in-activations.

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