Carboplatin loaded polymethylmethacrylate nano-particles in an adjunctive role in retinoblastoma: An animal trial

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Purpose: The purpose of the study is to compare the intra-vitreal concentrations of carboplatin, post peri-ocular injections of commercially available carboplatin (CAC) and a novel carboplatin loaded polymethylmethacrylate nanoparticulate carboplatin (NPC), in either eye, as a model system for treatment of advanced intra-ocular retinoblastoma (RB). Design: Experimental, comparative, animal study. Materials and Methods: Polymethylmethacrylate nanoparticles were prepared by free radical emulsion polymerization of methyl methacrylate in aqueous solution of carboplatin in the presence of surfactant sodium dodecyl sulfate and thermal initiator ammonium persulfate. 21 Sprague-Dawley rats, aged between 6 weeks and 3 months were enrolled. The right eye of each rat was injected peri-ocularly with CAC formulation (1 ml of 10 mg/ml) and the left eye with NPC (1 ml of 10 mg/ml), post-anesthesia, by an ophthalmologist trained in ocular oncology. Three rats each were euthanized on days 1, 3, 5, 7, 14, 28 and 42, post-injection and both eyes were carefully enucleated. Intra-vitreal concentrations of CAC and NPC were determined with Inductively Coupled Plasma Atomic Emission Spectroscopy. Analysis of data was done with paired t-test. Results: The intra-vitreal concentration of carboplatin with NPC was ~3-4 times higher than with CAC in all animals, on all the days (P < 0.05). Conclusion: A higher trans-scleral permeability gradient is obtained with the novel nanoparticles than with the commercial drug, leading to sustained higher levels of carboplatin in the vitreous. Peri-ocular injection of NPC could thus have an adjuvant efficacy in the treatment for advanced clinical RB, specifically those with vitreous seeds.

Key words: Carboplatin, intra-vitreal concentration, nanoparticle, peri-ocular injection, polymethylmethacrylate, retinoblastoma, subconjunctival

Retinoblastoma (RB) is a common tumor of infancy and childhood. Children with RB have been historically treated with external beam radiation therapy (EBRT) or enucleation or both. However, with the focus shifting to globe preserving measures and concerns over EBRT, systemic chemotherapy has become the mainstay of treatment in the past decade.[1-3] Chemotherapy has been shown to be effective in decreasing the size of the intraocular RB.[3,8] However, it is not without its associated drug related toxicities and risk of secondary neoplasm.[3] In addition, systemic intravenous chemotherapy alone has been shown to be less effective in tumors with vitreous seeding,[3,7] probably due to low levels of chemotherapeutic drugs achieved in the vitreous due to the blood retinal barrier. Thus, recent investigators have focused on evaluating high-dose, focal chemotherapeutic protocols to reduce these systemic side effects.[8-10]

Peri-ocular injection of commercially available carboplatin (CAC) has drawn much focus in the past decade. Peri-ocularly injected CAC has been reported to have a low efficacy when used alone.[11] However, studies have shown improved efficacy in the treatment of advanced intra-ocular RB, with adjunctive peri-ocular injection of carboplatin administered along with systemic chemotherapy.[11,12] It is believed that a high intracellular carboplatin level within tumor tissue is associated with increased tumor control.[13] This has led to postulates that if we could somehow further increase the intra-ocular concentration of carboplatin by injecting carboplatin via the peri-ocular route, it can not only be a good adjunct to intravenous systemic chemotherapy for intra-ocular RB, but may also be a potential option of actually decreasing, if not completely ameliorating the amounts of intra-venous chemotherapy the patients are currently subjected to.

With the advent of nanotechnology, polymeric biocompatible nanocarriers have emerged as a suitable vehicle for passive targeting of a drug to the site of action. The use of multifunctional nanostructures can prolong the half-life of drug circulation without increasing immunogenicity (as may occur in other carriers like bovine serum albumin or human serum albumin) and may reduce the side-effects and need for multiple injections due to their longer circulation time and sustained release behavior in vivo.[14] We performed a comparative animal trial in rats using peri-ocular injection of carboplatin loaded in a nanoparticle matrix of bovine serum albumin, which resulted in statistically significant increase in intra-vitreal carboplatin concentration in the 1st week post-treatment as compared to CAC, but the increased intra-ocular carboplatin levels were not sustained beyond the 1st week.[15] Although albumin (being a highly biodegradable and a hydrophilic carrier) resulted in a
high loading content of hydrophilic drug carboplatin, it was not able to maintain the therapeutic dose of carboplatin in vitreous beyond 1st week.

Acrylic polymers are known for their high biocompatibility within the eye (used thus far for various ocular implants manufacturing) and therefore may prove to be a better drug delivery vehicle in nanoparticulate form. Poly(methylmethacrylate) (PMMA), a hydrophobic, bio compatible and slowly biodegradable acrylic polymer, is a widely used drug delivery vehicle for antibiotics and non-steroidal anti-inflammatory drugs.[10] Hence, to eliminate any immunogenic effect and potentially achieve a sustained release system, we synthesized a novel PMMA carboplatin nanocarrier, with high drug loading efficiency. We then compared the time dependent intra-vitreal concentration of carboplatin after a single dose peri-ocular injection of CAC and a PMMA based nanoparticulate carboplatin (NPC), in a comparative animal trial.

Materials and Methods

This was an experimental, comparative, animal study conducted in 21 white Sprague-Dawley rats, aged between 6 weeks and 3 months. The authors confirm adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation and characterization of carboplatin loaded PMMA nanoparticles

PMMA nanoparticles were prepared by free radical emulsion polymerization of methyl methacrylate from the aqueous solution containing carboplatin in the presence of surfactant sodium dodecyl sulfate (SDS) and thermal initiator ammonium persulfate (APS).[17] Briefly, methylmethacrylate was added drop wise in an inert atmosphere of N₂ to a solution of SDS and excipient free carboplatin in Millipore water (50 ml) at 78-80°C in a 100 ml Schlenk flask fitted with thermometer and water condenser. The polymerization was initiated by slow addition of initiator APS at 78-80°C on a hot well plate for 24 h in N₂ atmosphere with high speed magnetic stirring followed by cooling to room temperature by stirring for 10 h at 25°C. The stock suspension of PMMA nanoparticles was purified by dialysis against de-ionized water with dialysis membrane (MW cut-off 12,400 Da) for another 24 h and stored at 4°C for further use. A part of the nanoparticles was lyophilized (at −54°C, 0.12 mbar) for 48 h to get free flowing powder and stored at −20°C for further studies. The analysis of particle size was done by dynamic light scattering (DLS) and surface charge by zeta-potential measurement. Binding and structure were analyzed by Fourier transform infrared spectroscopy and proton nuclear magnetic resonance (1H NMR) spectroscopy. Transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used for particle size, shape and surface roughness measurement and comparison. X-ray diffraction was used to measure retention of any drug crystalline behavior and stability of nanoparticle was assessed over a period of 1 month using DLS in deionized water, phosphate buffer saline (PBS: 7.4) and cell culture mediums.

Animal experiment

21 white Sprague-Dawley rats were anesthetized with halothane. The right eye of each rat was injected peri-ocularly with CAC. The left eye was injected peri-ocularly with the NPC. Both eyes were injected with 1 ml (10 mg/ml) of the chosen drug, in posterior subtenon’s (PST) location by the ophthalmologist (DS) with specialized training in ocular oncology, experienced in having performed similar injections in human patients with RB. Care was taken to avoid penetration of the globe and the globe was evaluated carefully post-injection. The animals were then revived and returned to their respective enclosures. Three animals each were euthanized at day 1, day 3, day 5, day 7, day 14, day 28 and day 42 and both eyes were enucleated by the ophthalmologist.

Methodology of injection technique

A volume of 1 ml of each formulations was injected with a 30 gauge needle via a PST route, taking special care to ensure against penetration of the globe. Egress of the carboplatin to the ocular surface was prevented by a cotton-tipped applicator placed at the conjunctival injection site, post-needle withdrawal.

Method of evaluating vitreous concentration

Post-enucleation, the eyes were stored in randomly numbered containers. The technician used a random number generator to store the 42 eyes. The complete eye was stored at −80°C in 500 μl of distilled water. The platinum content in vitreous was initially evaluated by a standardized high-performance liquid chromatography method for carboplatin using UV detector; each eye was sonicated in ice (for 8 min). Vitreous was extracted from the eye and vibromixed and centrifuged (20 min, 8000 RPM). Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used later for carboplatin estimation, which has been shown to be highly sensitive in tissue platinum detection.[18,19] The estimation of levels of carboplatin was achieved by measurement of platinum present in it by ICP-AES, (Ultima 2, Jobin Yvon Horiba, Japan) with plasma gas (argon) flow rate: 12 l/min, auxiliary gas flow rate: 0.2 l/min, sample uptake: 2.5 ml/min, integration time: 5 s. Organic content (if any) of the sample was decomposed using 60% nitric acid digestion at 120 ± 2°C for 6 h in closed polytetrafluoroethylene vials followed by cooling to room temperature and then diluting with distilled water prior to estimation.

Statistical analyses

The intra-vitreal concentrations of CAC and NPC were tabulated as mean ± standard deviation. Analysis of data was carried out with the paired t-test for each batch. P < 0.05 was considered statistically significant.

Results

The carboplatin loaded PMMA nanoparticles used in the study have nearly spherical shape with an average diameter of 110 ± 10 nm, as measured by DLS and confirmed by similar particle size obtained from TEM, SEM and AFM. However, by varying the preparation conditions, we could vary the size from 93 to 183 nm. The particles were nearly monodisperse with polydispersity index of <0.25. The surface charge on the nanoparticle was highly negative as obtained from zeta potential measurements. The in vitro release profile of carboplatin from PMMA nanoparticles followed a biphasic pattern, which established the sustained release behavior.

Post-injection of NPC in one eye and CAC in the other eye, the intra-vitreal carboplatin concentration obtained with
nano-particulate carboplatin and commercially available carboplatin migration initially in to the vitreous, compared to CAC, by transport of NPC over CAC is that the NPC, comprising of commercial drug. Our hypothesis for the increased trans-scleral gradient obtained with nanoparticles compared with the can be attributed to the higher trans-scleral permeability till 42 days. These sustained higher carboplatin drug levels concentrations of CAC in all the animals, as observed was significantly higher when compared with vitreous In the present study, vitreous concentration of NPC Migration of carboplatin in vitreous was ~3-4 times higher for NPC than CAC, on all the days. *Statistically significant. Values expressed in μg/g of vitreous; the values can be considered similar when expressed in μg/ml for comparison with literature data, since vitreous density is nearly equal to that of water.

**Table 1: Descriptive statistics (paired t test) and comparison between two formulations versus number of days**

| Days post injection | NPC (μg/g of vitreous) | CAC (μg/g of vitreous) | Difference of mean carboplatin obtained with NPC and CAC (μg/g of vitreous) (means±SD) |
|---------------------|-------------------------|-------------------------|----------------------------------------------------------------------------------|
|                     | Mean±SD (n=3) | Mean of total drug up to 42 days | SEM | 95% CI | Mean±SD | Mean of total drug up to 42 days | SEM | 95% CI | T (from paired t test, group wise) | P value |
| Day 1               | 261.6±47.9 | 317.2 | 27.7 (207.3, 315.9) | 74.0±1.2 | 85.8 | 0.7 (72.6, 75.4) | 187.6±46.7 | 6.77 | 0.010* |
| Day 3               | 272.6±37.2 | 317.2 | 21.5 (230.5, 314.8) | 77.6±12.3 | 2.0 (73.6, 81.6) | 227.8±47.9 | 7.66 | 0.008* |
| Day 5               | 305.4±51.4 | 29.7 (247.2, 363.7) | 77.6±3.5 | 7.1 (63.7, 91.5) | 195.0±24.9 | 9.06 | 0.005* |
| Day 7               | 318.8±93.0 | 53.7 (213.5, 424.1) | 99.7±4.3 | 2.5 (94.7, 104.7) | 219.1±88.6 | 4.07 | 0.027* |
| Day 14              | 333.3±38.4 | 22.2 (289.8, 376.9) | 85.8±5.7 | 3.3 (79.3, 92.3) | 247.5±32.7 | 11.13 | 0.003* |
| Day 28              | 350.2±33.8 | 19.5 (311.9, 388.5) | 98.1±11.7 | 6.8 (84.8, 111.5) | 252.0±22.0 | 12.89 | 0.002* |
| Day 42              | 378.1±36.6 | 21.1 (336.7, 419.6) | 87.9±21.9 | 12.6 (63.0, 112.7) | 290.2±14.6 | 13.71 | 0.002* |

Intra-vitreal carboplatin concentration was ~3-4 times higher for NPC than CAC, on all the days. *Statistically significant. Values expressed in μg/g of vitreous; the values can be considered similar when expressed in μg/ml for comparison with literature data, since vitreous density is nearly equal to that of water.

**Figure 1: Distribution of carboplatin in vitreous with the two formulations: nano-particulate carboplatin and commercially available carboplatin**

NPC was significantly higher than CAC, after 24 h (day 1). This trend continued and significantly higher intra-vitreal levels (~3-4 times) of carboplatin were obtained with NPC compared to CAC on days 3, 5, 7, 14, 28 and 42 as well, post-injection [Table 1 and Figure 1].

All the animals had mild chemosis in both eyes, immediately post the injection. However, the chemosis quickly decreased. There was no difference between the two eyes, either in the immediate post-injection phase or in the later stages.

**Discussion**

In the present study, vitreous concentration of NPC was significantly higher when compared with vitreous concentrations of CAC in all the animals, as observed till 42 days. These sustained higher carboplatin drug levels can be attributed to the higher trans-scleral permeability gradient obtained with nanoparticles compared with the commercial drug. Our hypothesis for the increased trans-scleral transport of NPC over CAC is that the NPC, comprising of macromolecule PMMA, establishes a stronger trans-scleral migration initially in to the vitreous, compared to CAC, by an uncertain mechanism. In addition, CAC may also get rapidly dispersed and cleared faster by lymph and/or blood circulation in the peri-ocular space than the drug embedded in the nanoparticles, thus allowing for prolonged retention of the NPC in the peri-ocular space. This hypothesis is supported by another study which showed that smaller particles are cleared much faster than the larger nanoparticles by the peri-ocular circulation.[20] Furthermore, the release of the drug from the NPC occurs by the erosion of the nanoparticle in due time course, thus contributing to its sustained release behavior kinetics.

Local delivery of chemotherapeutic agents increases the intra-ocular drug concentration and hence reaches the therapeutic window rapidly, which is possible by systemic therapy only with high dose. However, efficacy of peri-ocularly injected CAC, when used alone, is reported to be low in advanced RB, thus requiring multiple injections.[11,21] Use of multiple peri-ocular injections and rapid dispersion of the aqueous solution of carboplatin to the surrounding orbital tissue has been attributed to the development of local tissue toxicities such as orbital fat atrophy, ocular motility restriction,[22] optic nerve ischemic necrosis and atrophy[23] and preseptal cellulitis.[24,25] Thus, to reduce the potential number of injections needed (important as children require anesthesia each time), to prevent toxicities in the peri-ocular areas and to enhance the subconjunctival drug delivery, a sustained release system, which can improve the trans-scleral migration of carboplatin along with maintenance of the drug therapeutic window after a single dose injection, may be required. In addition, physicochemical characteristics of CAC, such as high solubility in water, high binding affinity to plasma proteins and degradability, may limit its therapeutic efficacy. These obstacles on its clinical application can be resolved by using small polymeric nanoparticles, which have unique characteristics of relatively large surface (functional) area which are able to bind, adsorb and electrostatically carry drugs, improve drug stability and release the native drug at a sustained rate at the target site.[14]

Subconjunctival injection of carboplatin in fibrin sealant demonstrated the advantage of sustained release behavior and minimal local toxicities in treatment of murine
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