In vitro Effects of Canine Wharton’s Jelly Mesenchymal Stromal Cells and Micellar Nanoparticles on Canine Osteosarcoma D17 Cell Viability

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Abstract

Objectives: To isolate and maintain canine Wharton’s jelly mesenchymal stromal cells (WJMSC) in culture, determine the effects of micellar nanoparticles containing doxorubicin (DOX) on WJMSC and canine osteosarcoma (OSA) D17 cells, and determine the effects of WJMSC loaded with micellar nanoparticles containing DOX on OSA D17 cell viability.

Procedures: Stromal cells were isolated from canine umbilical cords. Micellar nanoparticles containing DOX were prepared and added to individual culture plates containing canine WJMSC and OSA D17 cells to determine DOX in micelles (DOX-M) effects on cell growth and viability. Conditioned media (CM) from culture plates containing canine WJMSC incubated with various DOX-M concentrations was added to OSA D17 cells. An MTT assay was performed to assess osteosarcoma D17 cell viability. A trypan blue stain was utilized to perform cell counts to determine the effect of the DOX-M on WJMSC growth.

Results: WJMSC were successfully isolated and maintained in culture. Micellar nanoparticles containing DOX decreased viability of OSA D17 cells. Osteosarcoma D17 cell viability decreased following incubation with CM obtained from WJMSC loaded with DOX-M. Significant decreases in OSA D17 cell viability were observed after incubation with the CM of canine WJMSC loaded with 10 μM DOX-M at 96 hours (p<0.0037). Significant decreases were also observed with the CM from WJMSC loaded with 1 μM DOX-M at 96 hours (p<0.0222). WJMSC numbers decreased in a dose dependent manner following incubation with DOX-M. The decrease in WJMSC number was not secondary to cytotoxicity as all variables produced similar percentages of dead WJMSC.

Conclusions: Canine WJMSC can be isolated and maintained in culture, DOX-M produces OSA D17 cytotoxicity and slows proliferation of canine WJMSC. WJMSC containing DOX-M cause OSA D17 cell cytotoxicity. These data support in vivo experiments utilizing canine WJMSC and micellar nanoparticles.

Keywords: Nanoparticles; Osteosarcoma; Wharton’s jelly mesenchymal stromal cells; Micelles; Canine

Abbreviations: CM: Conditioned media; DM: Defined media; DOX: Doxorubicin; DOX-M: Doxorubicin in micelles; DPBS: Dulbecco’s Phosphate Buffered Saline; EGF: Epidermal Growth Factor; FBS: Fetal Bovine Serum; HPLC: High Performance Liquid Chromatography; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; NP: Nanoparticle; OSA: Osteosarcoma; PDGF: Platelet-derived Growth Factor; PEG: Polyethylene glycol; PES: Polyethersulfone; WJMSC: Wharton’s Jelly Mesenchymal Stromal Cells

Introduction

Osteosarcoma (OSA) is the most common primary bone tumor in dogs. Large and giant breed dogs that are middle aged to older are most commonly affected and the majority of tumors arise in the appendicular skeleton [1]. Many dogs afflicted with this disease die as a result of pulmonary metastasis.

Various chemotherapeutics have been investigated to treat canine OSA in the adjuvant setting. Efficacy has been observed with the platinum analogues cisplatin [2-6] and carboplatin [7,8] the anthracycline antibiotic doxorubicin (DOX) [9], and combinations of these drugs [10-14]. Patients receiving these agents frequently experience side effects that are at times severe. DOX is a commonly utilized drug in both human and veterinary medicine. Acute side effects that patients may experience include hypersensitivities, extravasation injuries, and arrhythmias. Gastrointestinal toxicity, myelosuppression, and cumulative cardiotoxicity may also occur [15,16].

Investigators are continually searching for ways to decrease chemotherapy side effects while still maintaining or even improving treatment efficacy. Nanoparticle (NP) systems have recently been discovered for use as novel cancer therapies in that they can be used as a drug delivery system to offer targeting of disease, enhanced drug delivery to and uptake by cells, improved drug solubility, continual drug release, reduced drug clearance, increased drug stability, reduced toxicity to non-target organs, and drug delivery across barriers. Micelles are one of many NP systems. Micelles consist of a hydrophobic core, which can serve as a reservoir for hydrophobic drugs, with a hydrophilic shell made up of polymers that stabilizes the core and makes the micelle water soluble.

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While passive disease targeting is observed with the use of NP, the efficacy of NP therapies may be improved if they are delivered directly to the target cells. One way to accomplish active tumor targeting is to utilize tumor-homing cells as delivery vehicles for nanotherapy payloads [17]. Wharton’s jelly mesenchymal stromal cells (WJMSC) are isolated from the Wharton’s jelly portion of the umbilical cord. Human WJMSC have been successfully used as an intravenous targeting vectors to deliver cytokines locally to metastatic tumors with subsequent attenuation [18, 19], and WJMSC isolated from rats have an intrinsic ability to migrate to and abolish rat mammary carcinoma after intravenous administration [20]. Utilizing WJMSC to deliver therapeutic agents housed in NP to a primary tumor and any metastatic foci that may be present could result in decreased systemic toxicity, decreased amount of drug necessary to achieve the same therapeutic effect, and an improvement in treatment response via an additive effect.

Data concerning stromal cell isolation from the Wharton’s jelly portion of canine umbilical cords is lacking. The first objective of this study was to isolate WJMSC from canine umbilical cords and propagate them utilizing cell culture methods. Investigations into utilizing NP in veterinary medicine are rare. Therefore, a second objective of this study was to report on the changes in OSA D17 cell viability after incubation with micellar NP containing DOX. The third and main objective of the study reported here was to determine what effects, if any, the combination of WJMSC and DOX in micelles (DOX-M) have on OSA D17 cell viability. The central hypothesis of this study was that canine WJMSC that have been loaded with micellar NP containing DOX will have a significant negative effect on canine OSA D17 cell viability in vitro.

Materials and Methods

Cell culture medium –Defined Medium (DM)
Consisting of expansion medium with 2% FBS and supplemented with growth factors (EGF, PDGF-BB) [21] was utilized for both WJMSC and OSA D17 cell lines.

Canine WJMSC: The WJMSC utilized in this study were isolated from an umbilical cord obtained during a caesarean section performed on an English Bulldog. The cord was refrigerated after harvest and during shipping, and was processed within 24 hours of receipt at Kansas State University. For processing, the umbilical cord vessels were manually removed and discarded. The remaining umbilical cord tissue was cut into smaller segments measuring three to five centimeters, rinsed with sterile saline, and placed into a Stomacher bag. An enzyme solution consisting of collagenase and hyaluronidase (collagenase type I: 200 units/mL, hyaluronidase from ovine testes 1 mg/mL CaCl2:3 mM; and DPBS) was then added to the bag. The Stomacher bag containing the umbilical cord material and enzyme solution was then placed into the Stomacher Biomaster 80 where it remained for 20 minutes. After maceration in the Stomacher, a 70 μm cell sieve was utilized to separate larger pieces of debris. The strained solution was then centrifuged at 1000 rpm for 3 minutes. The centrifuged pellet was resuspended in DM and seeded on a hyaluronic acid-coated flask and placed in an incubator at 37°C and 5% CO2. After 24 hours of incubation, cell flasks were rinsed, floating cells were removed, and the remaining adherent cells were expanded in DM. The number of passages that occurred prior to cells reaching replicative capacity was recorded.

Osteosarcoma Cells: Osteosarcoma D-17 cells were commercially obtained and maintained in DM at 37°C and 5% CO2. These cells represent an OSA cell line obtained from a pulmonary metastatic lesion in a poodle.

Nanoparticles: Micelles containing DOX were prepared as previously described, substituting DOX for amphotericin B [22]. Micelle formulation consisted of 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy(polyethylene glycol, Mn=3016 g/mol) (PEG-DSPE), cholesterol, and DOX. The PEG-DSPE (3.0 mg/mL in chloroform), cholesterol (0.4 mg/mL in chloroform) and DOX (0.29 mg/mL in methanol) solutions were mixed to obtain the desired PEG-DSPE: cholesterol ratio of 1:1:0.5. The mixture was then evaporated under a high vacuum to produce a thin film of coprecipitated drug and polymer. The film was dissolved in 10 mM HEPES, pH 7.0 and incubated at 50°C for 10 minutes to allow for equilibration. The solution was then filtered through a 0.45 μm PES syringe filter. The concentration of DOX was quantified by using a standard curve for DOX by HPLC.

After determination of DOX content of the micelles, they were prepared as a 1 mM concentration of DOX (i.e., all evaluations of these NP were subsequently based on amount of DOX contained within micelles, not some other parameter such as number of micelles). Serial dilutions were made to obtain DOX concentrations of 10 μM, 1 μM, 0.1 μM, 0.01 μM, and 0.001 μM within micelles.

Effects of the DOX-M on OSA D17 cells were determined by incubating DOX-M with OSA D17 cells. OSA D17 cells were plated at a density of 1700 cells/well in a 96 well plate. After 24 hours of incubation in DM, media was removed and media containing DOX-M at concentrations ranging from 10 μM to 0.001 μM was added. OSA D17 cells without DOX-M served as a negative control and OSA D17 cells incubated with free DOX at a concentration of 10 μM served as a positive control. At time points of 24, 48, 72, and 96 hours post DOX-M addition, an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) assay was performed, per the manufacturer’s protocol, to determine OSA D17 cell viability and proliferation. Each variable was evaluated in triplicate during three separate experiments. The results were averaged at each time point and plotted as percent of the control population.

Conditioned media experiments: Effects of WJMSC containing DOX-M on OSA D17 cell viability were determined via a conditioned media (CM) approach. The experiments were performed utilizing CM obtained from wells that contained canine WJMSC loaded with DOX-M.

To obtain the CM, canine WJMSC were plated at a density of 15,000 cells/well of a 24 well plate. After 24 hours of incubation, the media was removed and media containing micelles sufficient to give DOX concentrations ranging from 10 μM to 0.001 μM was added. After 24 hours of incubation, the WJMSC were viewed with a fluorescent microscope (TRITC filter, excitation BP 545/25, emission BP 605/70) to confirm loading of DOX-M within the WJMSC. After confirmation of loading, the media was removed and media without DOX-M was added. The WJMSC were allowed to incubate for 48 hours to allow the DM to become CM.

After confirmation of DOX-M loading into the WJMSC, OSA D17 cells were plated at a density of 1700 cells/well of a 96 well plate and allowed to incubate for 48 hours. The media was then removed from the OSA D17 cells and the CM from the WJMSC containing DOX-M was transferred to the OSA D17 cells. Wells containing only OSA D17 cells with fresh DM, OSA D17 cells with free DOX at a concentration of 10 μM, and OSA D17 cells with CM obtained from unloaded WJMSC served as controls. The MTT assay was performed every 24 hours for 96 hours. Each variable was evaluated in triplicate during three separate
DOX-M into WJMSC was confirmed (Figure 1B). Successful loading of DOX-M nanoparticles was observed with the treatment variables of 10 μM free DOX, and 10 μM and 1 μM DOX-M (Figure 2). Growth inhibition of greater than 80% was observed at 96 hours with the 10 μM free DOX and the 10 μM and 1 μM DOX-M (Figure 2). Growth inhibition of approximately 65% was observed at 96 hours with the 0.1 μM DOX-M (Figure 2). Growth inhibition was similar between free DOX and both the 10 μM and 1 μM DOX-M at 72 and 96 hours. The maximum growth inhibition at 24, 48, and 72 hours was observed with the 10 μM DOX-M and with both the 10 μM and 1 μM DOX-M at 96 hours. Both the 10 μM and 1 μM concentrations of DOX-M exhibited greater cytotoxicity than the 10 μM free DOX at the 72 and 96 hour time points.

Significant reductions in cell viability, when compared to the control OSA population, were found at 48 hours after incubation with 10 μM free DOX (p<0.0248), 10 μM DOX-M (p<0.0071), and 1 μM DOX-M (0.0368). OSA cell viability was also significantly decreased at 72 hours after incubation with 10 μM free DOX (p<0.0001), 10 μM DOX-M (p<0.0001), 1 μM DOX-M (p<0.0001), 0.1 μM DOX-M (p<0.0001), and 0.01 μM DOX-M (p<0.0003). At 96 hours, significant reductions in cell viability when compared to the OSA control population were found after incubation with 10 μM free DOX, 10 μM DOX-M, 1 μM DOX-M, 0.1 μM DOX-M and 0.01 μM DOX-M at 72 (p<0.0003 and <0.0001 respectively) and 96 hours (p=0.0001 for both DOX-M concentrations).

### Results

#### WJMSC isolation

WJMSC were successfully isolated from canine umbilical cords (Figure 1A). Healthy, viable WJMSC exhibited a typical mesenchymal cell appearance with elongated nuclei and stellate cytoplasm. When reaching their replicative limit, which was determined to be between five and ten passages for the cell line used, the cells became round or ovoid and detached from the flask. Canine WJMSC survived freezing and thawing. Both WJMSC and OSA D17 cell lines survived culture in DM.

#### Micellar nanoparticles

PEG-DSPE| cholesterol micelles with a narrow particle distribution and containing DOX were successfully formed. Successful loading of DOX-M into WJMSC was confirmed (Figure 1B).

#### Osteosarcoma D17 viability

DOX-M decreased OSA D17 cell viability. The greatest effect was observed with the treatment variables of 10 μM free DOX, and 10 μM and 1 μM DOX-M (Figure 2). Growth inhibition of greater than 80% was observed at 96 hours with the 10 μM free DOX and the 10 μM and 1 μM DOX-M (Figure 2). Growth inhibition of approximately 65% was observed at 96 hours with the 0.1 μM DOX-M (Figure 2). Growth inhibition was similar between free DOX and both the 10 μM and 1 μM DOX-M at 72 and 96 hours. The maximum growth inhibition at 24, 48, and 72 hours was observed with the 10 μM DOX-M and with both the 10 μM and 1 μM DOX-M at 96 hours. Both the 10 μM and 1 μM concentrations of DOX-M exhibited greater cytotoxicity than the 10 μM free DOX at the 72 and 96 hour time points.

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are classified as stromal cells, it is likely that they are analogous to the canine Whiton's jelly-derived mesenchymal stem cells recently described. Further study of the WJMSC presented here, including characterization by cytofluorimetric assay and investigation of cell differentiation ability, would be required to definitely prove that the WJMSC are in fact stem cells. These investigations may be the focus of future studies.

Micelles that contained DOX were successfully formed. This NP formulation of DOX improves drug solubility and provides a mechanism for direct tissue targeting. These qualities may improve the therapeutic efficacy of DOX by increasing the drug concentration in the cells and tissue of interest while decreasing, or eliminating, the presence of the drug in normal cells.

Even though a NP formulation of a drug has the capability to accumulate in a diseased area, it is only of use if it also causes cytotoxicity to abnormal cells. The results of this study provide evidence that micellar NP containing DOX have cytotoxic effects on OSA D17 cells in vitro. The greatest effect was observed in OSA D17 cells incubated with 10 μM and 1 μM DOX-M at 96 hours; however, the 10 μM free DOX exhibited similar results at the 96 hour time point. An interesting finding was that both the 10 μM and 1 μM concentrations of DOX-M exhibited greater cytotoxicity than the free DOX at the 72 and 96 hour time points. These results indicate that encapsulation into micelles increases rather than decreases the cytotoxicity of DOX and that DOX-M may be a good substitute for free DOX as similar cytotoxic effects were observed. Further studies should be performed to determine if this observation remains present in vivo.

The homing capability exhibited by WJMSC is believed to be due to chemokines and growth factors that are secreted by a tumor and its associated stroma [18, 24, 25]. It has been shown that once injected mesenchymal stem cells incorporate into lung tissue within three days [26]. As OSA predominantly metastasizes to lung and homing of WJMSC should occur in a similar manner and time frame as mesenchymal stem cells, WJMSC preloaded with the DOX-M payload could be an ideal treatment. The antiproliferative effects of DOX-M on the WJMSC observed in this study should not inhibit tissue targeting in vivo. As long as the WJMSC survive for this 72 hour period, allowing the cells to localize in diseased areas, they will then be in the optimal location to undergo cell death and release the DOX-M in the target area.

Even though WJMSC containing DOX-M have cytotoxic effects on OSA D17 cells in vitro. The decrease in OSA D17 cell viability observed with WJMSC plus DOX-M was similar to that observed with DOX-M alone. These findings indicate that loading the NP into the WJMSC does not diminish cytotoxicity. In fact, in an in vivo situation, the cytotoxic effects may actually be enhanced with the combined use of canine WJMSC and DOX-M. These results are promising and indicate the need for further study into DOX micelle and WJMSC usefulness in treating canine OSA.

The CM from the WJMSC containing the two highest concentrations of DOX-M, 10 μM and 1 μM, exhibited similar cytotoxic effects when compared to the 10 μM free DOX. These findings indicate that further study should focus on determining if the effects observed with the 10 μM and 1 μM DOX-M in vitro remain true in vivo.

The overall conclusion from these data indicate that DOX-M are cytotoxic to OSA D17 cells and that cytotoxicity may be enhanced with the combined use of canine WJMSC and DOX-M. While much work still needs to be done, these data indicate that WJMSC and DOX-M

| Variable       | Percent Dead WJMSC |
|----------------|---------------------|
| Unloaded WJMSC | 7.28%               |
| 10 μM DOX-M    | 13.89%              |
| 1 μM DOX-M     | 6.76%               |
| 0.1 μM DOX-M   | 4.65%               |
| 0.01 μM DOX-M  | 7.55%               |
| 0.001 μM DOX-M | 2.49%               |

Table 1: Percent of dead canine WJMSC post 24 hour incubation with DOX-M.
may provide a clinically relevant therapeutic option for OSA in canine and human patients in the future.

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