IN VITRO EFFECTS OF CANINE WHARTON'S JELLY MESENCHYMAL STROMAL CELLS AND NANOPARTICLES ON CANINE OSTEOSARCOMA D17 CELL VIABILITY

by

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Abstract

Objectives – To isolate and maintain canine Wharton's jelly mesenchymal stromal cells (WJMSCs) in culture, to determine the effects of micellar nanoparticles containing doxorubicin (DOX) on WJMSCs and canine osteosarcoma (OSA) D17 cell viability, and to determine the effects of conditioned media from WJMSCs loaded with micellar nanoparticles containing DOX on OSA D17 cell viability.

Sample Population – Canine WJMSCs containing various concentrations of DOX micelles and canine OSA D17 cells.

Procedures – WJMSCs were isolated from canine umbilical cords. Micellar nanoparticles containing DOX were prepared and added to culture plates containing canine OSA D17 cells to determine micelle effects on cell growth and viability. Conditioned media from culture plates containing canine WJMSCs incubated with various DOX micelle concentrations was added to OSA D17 cells for conditioned media experiments. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to assess OSA D17 cell viability. A trypan blue stain was also utilized to perform cell counts to determine the effect of the DOX micelles on stromal cell growth.

Results – WJMSCs were successfully isolated and maintained in culture. Micellar nanoparticles containing DOX decreased OSA D17 cell viability. OSA D17 cell viability was also decreased following incubation with conditioned media from canine WJMSCs loaded with micellar nanoparticles containing DOX. Significant decreases with the conditioned media of canine WJMSCs loaded with 10μM micelles occurred at 48 hours ($p < 0.005$) and at 72 and 96 hours ($p < 0.0001$). Significant decreases were also observed with the 1 μM DOX micelles at 72

hours ($p < 0.005$) and 96 hours ($p < 0.0001$). WJMSC numbers decreased in a dose dependent manner following incubation with DOX micelles. Changes in WJMSC number was not caused by increased cell death as all variables produced similar percentages of dead cells.

Conclusions – Canine WJMSCs were successfully isolated and maintained in culture. Stromal cells containing DOX micellar nanoparticles induced OSA D17 cell cytotoxicity while inducing an anti-proliferative, rather than cytotoxic effect, on the WJMSC. These data support future *in vivo* experiments utilizing canine WJMSCs and micellar nanoparticles.

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List of Abbreviations

AmB – amphotericin B

AUC – area under the curve

BBB – blood brain barrier

CM – conditioned media

DDS – drug delivery system

DM – defined medium

DMEM – Dulbecco's modified Eagle medium

DMSO – dimethyl sulfoxide

DOX – doxorubicin

DPBS – Dulbecco's phosphate buffered saline

EGF – epidermal growth factor

EPR – enhanced permeation and retention

FBS – fetal bovine serum

HPLC – high performance liquid chromatography

MDR – multidrug resistance

MSC – mesenchymal stromal cells

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

NPs – nanoparticles

OSA – osteosarcoma

PDGF – platelet derived growth factor

PEG – poly(ethyleneglycol)

PES - polyethersulfone

PMs – polymeric micelles

PPES – palmar plantar erythrodysesthesia

RES – reticular endothelial system (AKA mononuclear phagocytic system)

TNF-α – tumor necrosis factor alpha

UCB – umbilical cord blood

WJ – Wharton's Jelly

WJMSCs – Wharton's jelly mesenchymal stromal cells

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Dedication

This thesis is dedicated first and foremost to my husband, Patrick Reeds. His encouragement and belief in me is what has made all of my accomplishments possible. I would also like to dedicate this thesis to all my family, friends, and educators. You have all given me a strong foundation of knowledge and love that will continue to support me.

CHAPTER 1 - Significance of Research and Literature Review

Significance of Research Project

Cancer remains one of the leading causes of morbidity and mortality in both human and veterinary medicine. As the behavior of osteosarcoma is similar between dogs and humans, advances made in dogs with OSA may be translated into human medicine. This may allow our canine companions to improve the quality of care we receive.

Investigators are continually searching for ways to decrease chemotherapy side effects while still maintaining or even improving treatment efficacy. Utilizing stromal cells to deliver therapeutic agents housed in nanoparticles directly to a primary tumor and any metastatic foci that may be present is an attractive alternative. Decreased toxicity may be observed with this combination approach as targeted drug delivery may reduce or even eliminate many of the side effects that are observed with systemic administration. Other advantages include the use of lower drug dosages and the possibility that the combination of these two novel treatments may have an additive effect.

Literature Review

Osteosarcoma

Osteosarcoma (OSA), a malignant tumor of bone, is the most common primary bone tumor to occur in both humans and canines [1, 2]. OSA in humans frequently affects children and adolescents between the ages of 10-20 years with a second, smaller incidence peak occurring in those older than 60 years [1, 3]. The incidence of OSA is similar in dogs in that a double peak is observed; however, the smaller peak occurs in dogs aged 18-24 months with the larger peak

occurring in middle aged dogs with the median reported to be 7 years [2]. Large and giant breed dogs, such as Saint Bernards, Great Danes, and Rottweilers are most commonly affected. Dogs weighing over 40kg account for 29% of OSA cases while those weighing less than 15kg account for only 5% [4].

In both species, tumors most often occur in the metaphysis of long bones of the appendicular skeleton. Common locations include the distal femur, the proximal tibia, or the proximal humerus [2, 5-7]. Tumors may also occur in the bones of the axial skeleton, such as the skull, scapula, or rib. In dogs, tumor location is related to size while in humans it is related to age. Fifty-nine percent of OSA cases involving the axial skeleton occur in dogs weighing less than 15 kilograms [4] while in humans the majority of axial skeletal tumors are observed in elderly patients [1].

OSA is an aggressive malignancy and metastasis to the lungs, also known as relapse, is commonly observed in dogs and humans [8]. It has been reported that approximately 90% of dogs have pulmonary micrometastases at the time of OSA diagnosis [9]. Many dogs die or are euthanized due to metastatic disease progression and the development of radiographically visible pulmonary metastases. Other factors that may influence an owner's decision to euthanize include an inability to provide adequate pain relief without amputation of the affected limb or fracture of the primary tumor site.

Treatment for OSA in humans involves neoadjuvant chemotherapy combined with surgery [3]. Limb sparing procedures are commonly utilized, with a minority of humans requiring amputation. Unfortunately, the prognosis for this disease has not changed significantly in the last 20 years. Currently, 65% of patients are estimated to survive 5 years with an estimated relapse rate of 30-40% within 3 years [8].

The current standard of care to target the primary tumor and prolong a dog's life is limb amputation combined with adjuvant chemotherapy. This combination approach is effective at increasing survival times by slowing the progression of metastatic disease when compared to amputation alone. Patients that receive an amputation and adjuvant chemotherapy are expected to live approximately 8-12 months while those that receive an amputation alone generally survive only 6 months [7, 10-12]. Palliative options that are available for primary appendicular OSA include amputation alone, radiation therapy, bisphosphonate administration, oral pain medications, or a combination of these modalities. These therapies will not significantly extend the life of the patient past what is expected for amputation alone and most will eventually succumb to metastasis progression.

Chemotherapy

Chemotherapy is defined as the treatment of disease with chemicals. Cancer chemotherapy utilizes antineoplastic drugs to treat malignancies and is generally utilized as a systemic therapy. In general, chemotherapy targets rapidly dividing cells, which include both the neoplastic and the normal cells throughout the body. The most common side effects of chemotherapy occur in tissues that are made up of rapidly dividing cells such as the skin, the gastrointestinal tract, and the bone marrow. These side effects typically involve alopecia, diarrhea and/or vomiting, and decreased blood cell counts due to bone marrow suppression. Each cancer chemotherapy drug category has a specific mechanism of action to kill cancer cells; however, the overall goal is to damage DNA, prevent cancer cell replication, or induce apoptosis. Cancer chemotherapy may be utilized in the neoadjuvant or adjuvant settings or used alone in cases with chemotherapy responsive tumors.

Many human patients with OSA are treated in the neoadjuvant setting in which they receive chemotherapy prior to surgical intervention. People with high-grade OSA who respond well to neoadjuvant chemotherapy may continue to receive chemotherapy after surgery, known as the adjuvant setting. Chemotherapy drugs that have been utilized either alone or in combination for human OSA include methotrexate [13], doxorubicin (DOX) [14], cisplatin or carboplatin [15], ifosfamide or cyclophosphamide [16] actinomycin D, and bleomycin [17]. These drugs are typically given on an aggressive schedule with high drug dosages and short administration intervals prescribed.

Canine patients with OSA typically receive chemotherapy in the adjuvant setting as neoadjuvant chemotherapy has not been shown to improve response rates or survival times [18]. Many different chemotherapeutics have been investigated to treat canine OSA and efficacy has been observed with the platinum analogues cisplatin [7, 11, 19-21] and carboplatin [18, 22], the anthracycline antibiotic DOX [19], and combinations of these drugs [10, 12, 23-25]. In general, veterinary patients experience fewer and less severe side effects as compared to humans because they receive lower drug dosages on less aggressive administration schedules. However, the development of these effects may lead to discontinuation of therapy. Dogs receiving cisplatin may potentially experience nephrotoxicity, gastrointestinal toxicity, or myelosuppression [26, 27]. Intense saline diuresis protocols [28] are instituted before, during, and after cisplatin administration to help prevent nephrotoxicity. Carboplatin is similar to cisplatin, however, dogs experience fewer adverse effects with carboplatin compared to cisplatin and saline diuresis is not required for carboplatin administration as nephrotoxicity is not a concern. Possible side effects related to the administration of carboplatin include myelosuppression and gastrointestinal toxicity [29, 30].

Doxorubicin

DOX is an anti-neoplastic chemotherapy agent that is commonly utilized in human and veterinary oncology. It has a broad range of efficacy and is used to treat many tumors such as lymphoma, sarcomas, and even some carcinomas. DOX is classified as an anthracycline antibiotic. It was originally synthesized as an analogue to daunorubicin, the original anthracycline antibiotic, and both agents are derived from *Streptomyces*, a species of yeast [31].

DOX has several mechanisms of action, the main one being interference with topoisomerase II [31, 32]. Topoisomerase is an enzyme that relaxes super coiled double-stranded DNA so that it can be replicated and RNA can be transcribed. It also plays a critical role in chromatin condensation. Topoisomerase I forms a single strand nick in the DNA helix while topoisomerase II forms a double strand nick. These nicks relieve the torsional strain of the helix which then allows the DNA strand to swivel and be religated. DOX binds to and stabilizes the DNA-topoisomerase complex which prevents DNA religation. The irreversible damage to the DNA results when a DNA replication fork encounters the stabilized complex. This damage leads to lethal double strand breaks and cell death [31, 32].

DOX also intercalates into DNA, which can cause partial winding of the helix. This intercalation is not considered to be a main mechanism of action because the majority of the cell DNA exists as chromatin, which may provide partial protection from intercalation [31, 32]. Other mechanisms of action of DOX are formation of free radicals through metabolism of its quinone ring structure and production of superoxide (O^{2-}), and damage to the cell membrane [31, 32].

Canine patients receiving DOX experience a wide range of toxicities. Acute side effects that patients may experience include hypersensitivities, extravasation injuries, and arrhythmias [33]. Late side effects, such as gastrointestinal toxicity, myelosuppression, and cumulative cardiotoxicity, may also occur [27, 34].

Umbilical Cord Stromal Cells

Stem cells, whether obtained from fetal or adult tissue, have many desired characteristics that can be employed for cancer treatment. Two of these are the abilities to specifically home to an area of disease when injected systemically and to deliver therapeutic agents [35]. Being able to combine these two characteristics makes stem cells an attractive option for targeted cancer treatment. Unfortunately, the use of stem cells, especially those obtained from fetal tissue, is associated with much controversy and the search for viable alternatives is ongoing.

The umbilical cord is an abundant source of mesenchymal stromal cells (MSC). Various portions of the umbilical cord such as the umbilical cord blood (UCB), the umbilical vein subendothelium, and the connective tissue portion of the umbilical cord known as Wharton's jelly (WJ) have all been utilized as a source of MSCs [36].

Wharton's jelly mesenchymal stromal cells (WJMSCs) are isolated from the WJ portion of the umbilical cord). WJ is the connective tissue portion of the umbilical cord that surrounds the two umbilical arteries and the umbilical vein. The predominant makeup of WJ is glycosaminoglycans, the most abundant of which is hyaluronic acid. The presence of hyaluronic acid is important as it maintains the umbilical cord structure by protecting it from pressure [37]. Originally, WJMSCS were obtained from umbilical cords obtained from pigs [38]; however, isolation from humans [39] and various other species has since occurred [40, 41].

WJMSCs exhibit many of the same characteristics as embryonic stem cells; however, their use is much less controversial as the umbilical cord is a non-embryonic tissue that is generally discarded. Advantages of using the umbilical cord to obtain matrix stromal cells versus the bone marrow or other adult tissues include the large number of cells obtained with isolation [42], successful isolation from approximately 100% of samples [43], non-invasive harvest of the tissue and cells following the birth of the fetus, and the abundant supply of umbilical cords [44].

WJMSCs possess many desirable characteristics. They express genes characteristic of primitive stem cells, such as c-kit, [35, 36, 39] and they possess the abilities to migrate to an area of disease and deliver a therapeutic agent [35, 36, 44, 45]. WJMSCs are also multipotent, express telomerase, and express surface markers that are characteristic of mesenchymal stromal cells, including positive expression of CD10 and CD44 and negative expression of CD14 and CD45 [36]. Researchers have shown advantages over mesenchymal stem cells in that WJMSCs exhibit improved tissue repair and engineering, have shorter doubling times, and can be propagated for a greater number of passages before senescence is reached [35]. Taken together, these characteristics indicate that WJMSCs cells may have superior *ex vivo* expansion capabilities compared to other mesenchymal stromal cells.

Another advantage of the use of WJMSCs over stem cells is the lack of tumor formation. Undifferentiated embryonic stem cells that are transplanted will sometimes form tumors, most often teratomas [36]. To date, tumor formation has not been observed with the transplantation of WJMSCs [44].

Nanoparticles

A nanoparticle is a submicron-sized polymeric colloidal particle with diameters ranging from 10 to 100 nanometers. Nanoparticles (NPs) may be known by other names such as ultrafine particles, nanocrystals, quantum dots, or nanotubes. They have specific properties such as high surface to mass ratio, high solubility, high penetration power, high efficiency, high intracellular uptake, and the ability to carry compounds such as drugs, DNA, or proteins [46-48] that make them ideal to use for the diagnosis and treatment of disease. Possible applications for NPs in medicine include drug delivery, diagnostic testing, and production of better biocompatible materials [46].

In the field of oncology, NPs are most commonly utilized as a drug delivery system (DDS). Cytotoxic drugs do not differentiate between a cancerous cell and a normal cell, which leads to the adverse effects experienced by the patient. These toxicities limit the dose that may be administered; however, large quantities of drug are often required to overcome the rapid elimination and widespread distribution into normal organs and tissues that is observed with most anticancer drugs. Nanotechnology offers a targeted tumor approach and may provide significant benefits to cancer patients [49] as the intracellular concentration of the drug can be enhanced while avoiding toxicity in normal tissue.

There are many different types of NPs that can be utilized as a DDS including polymer-drug conjugates, dendrimers, polymer micelles, and liposomes. Therapeutic agents may be encapsulated, covalently attached, or adsorbed onto the NP of choice. The goals of utilizing NPs as a DDS include enhanced delivery to and uptake by target cells, disease targeting/homing, improved drug solubility, constant drug release, reduced drug clearance, increased drug stability, reduction in the toxicity of the free drug to non-target organs, and drug delivery across barriers

such as the blood brain barrier (BBB) [46, 50]. Utilizing NPs as a DDS may improve the safety and bioavailability of the anticancer drug while also increasing the therapeutic index.

Disease Targeting

A nanoscale DDS has the ability to travel to and act preferentially at a selected target, such as a tumor or metastatic foci, via active or passive targeting. Active targeting, which can occur only after the NPs have passively accumulated in the tumor [51], can be accomplished via the use of ligands conjugated to the surface of the NPs that recognize a specific receptor or epitope of the target tissue that is not found on normal tissue [50]. Ideally, the target should be expressed homogenously on the target cells. Targeting ligands may be monoclonal antibodies, antibody fragments, or non-antibody ligands and the ligand binding affinity influences tumor penetration [51].

More commonly, NPs accumulate in diseased tissue due to passive targeting. Passive targeting is frequently accomplished via enhanced permeation and retention (EPR) which occurs as a consequence of the abnormal vasculature present in cancerous tissues and results in high drug concentrations in the local area [50]. The angiogenesis that is required for tumor growth produces abnormal vessels that have large fenestrations, are very tortuous, lack a smooth muscle layer and pericytes, lack a constant blood flow and direction, lack efficient lymphatic drainage, and exhibit slow venous return [49, 51]. The large fenestrations allow for the NPs to accumulate in the extravascular space within the tumor and then diffuse into the neoplastic cell. Once inside the tumor, the NPs are trapped as the absence or decreased function of lymphatic vessels prevents NP drainage [51]. This local accumulation may lead to enhanced tumor cytotoxicity

and improvement in the therapeutic index [46] which will allow for lower amounts of drug to produce the same effect while decreasing toxicity [50].

Passive targeting and EPR also occur due to the tumor microenvironment. Cancerous cells exhibit rapid growth and increased metabolic rates that require high amounts of oxygen and nutrients. This demand is met via glycolysis, which results in an acidic tumor environment. The NPs are designed to be stable at a physiologic pH of 7.4 and to release active drug in the target tissue with lower pH values [52], meaning that the drug will remain within the NP carrier while in the systemic circulation and be released once it reaches the target tissue.

Other passive targeting strategies are tumor-activated prodrug therapy and direct local delivery of anticancer agents to tumors. With tumor activated prodrug therapy, the drug is conjugated to a tumor specific molecule and remains inactive until it reaches its target. Direct tumor injection, multiple injections or surgical procedures are utilized to deposit the active anticancer drug directly at the target site. This approach eliminates the need for systemic drug administration; however, the procedure is invasive and some tumors are difficult to access [49].

Improved Drug Solubility

One of the main reasons to utilize NPs as a DDS is to improve the poor solubility of many anti-neoplastic drugs. Most anticancer drugs are lipophilic and therefore poorly soluble in water [49, 53] which results in low bioavailability. Emboli may form when the drugs are administered intravenously and drug accumulations in specific areas may cause increased local tissue toxicity. A drug's solubility is automatically increased when encapsulated within the hydrophobic core of the NP [53]. Developing a new drug to overcome solubility problems would be time consuming and expensive. Utilizing NPs as a DDS would help increase drug

bioavailability, decrease normal tissue toxicity, and prevent drug degradation [54] in a much faster and cost effective manner.

Reduced Drug Clearance

There are normal barriers present in the body that make the size and charge of a NP crucial in determining its fate. The kidneys are able to freely filter particles that are less than 10 nm in diameter and will also remove particles that carry a positive charge. Particles that are larger than 100 nm are removed by the liver and the reticular endothelial system (RES) [47]. The RES consists of macrophages located in the liver and spleen that act as phagocytic scavengers that remove the NP DDS from the systemic circulation thereby preventing delivery to tissues other than the liver and spleen [47, 50]. With these limitations in mind, an ideal NP for use as a DDS should be between 10 and 100 nm, carry a neutral or anionic charge, and must be hidden from the RES [51]. Surface modification of the NP DDS via the addition of hydrophilic polymers or the attachment of poly(ethyleneglycol) (PEG) chains will decrease opsonization of the NP by the immune system. The decreased opsonization then prevents clearance by the kidneys and the RES [47, 55], thereby allowing the NP DDS to circulate in the body for a prolonged period of time.

Drug Delivery Across Barriers

The small size of NPs allows for cellular uptake and transport through biological barriers. One of the most important barriers in the body is the BBB. Most drugs are not able to traverse this barrier in a normal patient as the junctions between endothelial cells and astrocytes are very tight. The BBB is likely abnormal in patients with intracranial disease, which may allow passage of drugs and in normal animals drugs may pass via endogenous BBB transporters such as carrier

mediated transport, active efflux transport, or receptor mediated transport [46]. It has been shown that NP DDS are capable of passing through the BBB. This passage is possibly due to cerebral endothelial toxicity that is caused by the NP but the more likely explanation is that the NP formulation allows for decreased drug binding to the p-glycoprotein cellular efflux system. [46].

Bypassing the p-glycoprotein efflux system not only allows drug passage into an otherwise non-admissible area, but it may also provide the advantage of decreasing chemotherapy resistance mediated through multidrug resistance (MDR) [50]. MDR may induce chemotherapy resistance via increased drug metabolism, increased drug transporters and efflux proteins such as p-glycoprotein, and the development of point mutations in drug targets [50]. There are many drugs that are susceptible to MDR including DOX, vincristine, and vinblastine. Research has shown that encapsulating these drugs in liposomes, for example liposome encapsulated DOX known as Doxil®, allows the drug to maintain efficacy even when MDR is present [56]. The NPs are able to bind to the cell membrane and enter the cytoplasm via receptor-mediated endocytosis. This allows the NPs to avoid detection and expulsion by p-glycoprotein [52].

Problems with Drug Delivery Systems

Though NP DDS have their advantages and appear to be ideal forms for *in vivo* cancer treatments, several challenges exist. As the NPs are very small, they can only hold a limited amount of drug. Larger amounts of drug could be used, but the tradeoff would be increased NP size and possible aggregation [53]. The small size of the NPs may also lead to a faster release of drug, which may cause problems if the NP has not yet reached the intended site.

One aspect of the use of NPs as DDS that has not been fully investigated is the possibility of toxicity. NPs are not like other materials that are produced in bulk as they are developed for their unique surface properties. The NP surface comes into contact with tissue in the body and determines the particle response. As such, the unique composition of the NPs utilized need to undergo toxicity studies [46]. These investigations should focus on NPs combined with the drug of interest and "empty" NPs that do not contain the drug. This is especially important when investigating non degradable particles as their constant presence in the body may lead to chronic inflammatory reactions. There is also a lack of understanding of the basic biologic behavior of the NPs at the organ and cellular levels *in vivo*. Therefore, pharmacokinetic and distribution studies of the NPs are needed [46].

Some toxicities of NPs have been reported. Studies performed *in vivo* have shown that NPs are capable of causing pulmonary inflammation in rats and that they affect the immune response to common allergens [46]. Toxicities that have been found *in vitro* include inhibition of macrophage phagocytosis, platelet aggregation, and adverse effects on cardiac function and vascular homeostasis [46]. These toxicities were usually only found with specific NPs indicating the need to determine the full spectrum of toxicities for each NP as new toxicities may be observed while other previously observed toxicities may be enhanced.

Many researchers have reported the use of NPs as a DDS in human medicine. Polymeric NPs have been synthesized to deliver paclitaxel [57], gold NPs have been utilized to deliver tumor necrosis factor-alpha (TNF-α) to solid tumors [58], and liposomes have been utilized to encapsulate DOX to help decrease cardiotoxicity [50] and improve treatment efficacy in people with Kaposi's sarcoma [59], recurrent/refractory ovarian cancer [60], and other cancers [61].

The use of NPs in veterinary medicine is significantly behind that of human medicine. One NP based DDS that is utilized in veterinary medicine is a liposomal formulation of DOX known as Doxil. Doxil has been studied in dogs with hemangiosarcoma [62] and cats with soft tissue sarcomas [63, 64]. Unfortunately, the use of Doxil has been associated with severe toxicity and the benefit to risk ratio does not indicate that this is currently a viable option. Further, Doxil is expensive and without the help of medical insurance, it is unaffordable for many veterinary clients. These limitations indicate that newer approaches utilizing NPs in veterinary medicine are needed.

Polymeric Micelles

An ideal DDS should spontaneously form from the mixture of drug molecules and carrier components, have a size of 10-100 nm for extravasation and tissue penetration, remain stable *in vivo* for a long period of time, cause no biological side effects, release the therapeutic component when in contact with the target tissue, and the carrier components should be easily cleared from the body once their therapeutic function is completed [65]. Polymeric micelles (PMs), the focus of the research presented here, meet all these criteria as they are small and possess the ability to solubilize hydrophobic drugs or imaging agents. PMs are also biocompatible, self-assembling, possess targeting potential and due to their pharmacokinetics, they have the ability to improve tumor cell kill with reduced systemic toxicity [52, 53].

PMs are spheroid NPs that are formed from block or graft copolymers in which drugs can be incorporated into via chemical conjugation or physical entrapment [66]. The PM structure consists of an inner core and an outer shell. The core is hydrophobic and is utilized to encapsulate drugs that are poorly water soluble, thereby increasing their water solubility. The

shell is hydrophilic and protects the encapsulated drug from the aqueous environment in the body. The shell allows a long circulation time as the PMs are able to prevent opsonization, thereby avoiding detection by the RES *in vivo*. The RES would normally remove the micelles from circulation, rendering them useless for disease treatment [67].

It has been shown that PMs are able to specifically localize in tumor tissue due to the EPR effect [65-67]. Once the PMs have reached their target, they are internalized into cells via fluid-state endocytosis, a process that occurs in the absence of a surface ligand for binding [67]. Once inside the cell, the drug is released. The mechanism depends on the method utilized to encapsulate the drug, the amount of drug loaded into the micelle, the length of the core-forming part of the polymer, and the presence of cross-linking within the core. Bulk degradation of the polymer matrix, surface erosion, or drug diffusion are all possible methods of release [67].

While still fairly new as a DDS, PM formulations are currently under investigation. Genexol®-PM (PEG-poly(D,L-lactide)-paclitaxel), a cremophor-free polymeric micelle-formulated paclitaxel, is currently undergoing clinical trials [68] as the first commercially available polymeric formulation of an anti-cancer drug, [52]. Paclitaxel is a taxane derived from the Pacific yew tree that has a wide spectrum of antitumor activity. In humans, the drug is used to treat cancers of the breast, ovary, head and neck, and lungs. A formulation of paclitaxel, Taxol, is poorly soluble in water and requires a surfactant called Cremophor EL to improve its solubility. The addition of Cremophor EL induces substantial toxicity such as severe hypersensitivity reactions. Through the use of the block copolymer system in Genexol®-PM, this toxicity is greatly reduced while the solubilization of paclitaxel is maintained [68].

PMs have also been investigated for use with anti-fungal agents. Recently, PMs were utilized to solubilize amphotericin B (AmB) to reduce its toxicity while maintaining antifungal

activity [69]. Cholesterol was incorporated into the outer shell of the PMs to determine if its presence could help slow delivery of the AmB and reduce the toxic side effects commonly observed with its use. The results of this study showed that the incorporation of cholesterol resulted in sustained release of AmB *in vivo* when compared to PMs that did not contain cholesterol. These data support the use of cholesterol in the production of PMs, and are the basis for the PMs engineered and investigated in this study.

CHAPTER 2 - Chapter 2: Isolation and Maintenance of Wharton's Jelly Mesenchymal Stromal Cells from Canine Umbilical Cords

Hypothesis

Wharton's jelly mesenchymal stromal cells can be isolated from canine umbilical cords and maintained in culture.

Materials and Methods

Cell Culture Medium

Defined medium (DM) consisting of expansion medium with 2% serum and growth factors [70] was utilized for both WJMSC and OSA D17 cell lines for all experiments. Expansion medium consisted of 56% low-glucose DMEM[a], 37% MCDB-201[b], 1X insulin-transferrin-selenium[a], 1X AlbuMax I[a], dexamethasone 10^{-4}M in DMSO [b], 1% ascorbic acid 2-phosphate[b], 1X Pen/Strep 100X[a], and 2% fetal bovine serum (FBS)[c]. The expansion medium was supplemented with 100 μg/mL epidermal growth factor (EGF)[d], and 50 μg/mL platelet derived growth factor (PDGF-BB)[d].

Canine WJMSCs

Canine umbilical cords were obtained after a Caesarean section procedure. The umbilical cords were submitted by veterinarians throughout Kansas and from the Kansas State University Veterinary Teaching Hospital. The WJMSCs utilized in this study were isolated from an umbilical cord obtained from an English bulldog that underwent a Caesarean section in Beloit, KS.

After obtaining a whole umbilical cord, the umbilical cord vessels were manually removed and discarded. The remaining umbilical cord tissue was cut into smaller segments of 3 x 5 cm if needed and was then rinsed with sterile saline and placed into a Stomacher bag. An enzyme solution consisting of a hyaluronidase and collagenase (collagenase type I: 200 units/mL[a] hyaluronidase from ovine testes 1mg/mL[e] $CaCl_2$:3mM; and Dulbecco's phosphate buffered saline (DPBS)[a]) 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[f] where it remained for 20 minutes. After maceration in the Stomacher, a 70 μm cell sieve was utilized to separate out larger pieces of debris. The strained solution was then centrifuged at 1000 rpm for 3 minutes. Two thirds of the centrifuged pellet was resuspended in DM on a hyaluronic acid-coated plate. The remaining 1/3 of the pellet was resuspended in 90% FBS, 10% dimethyl sulfoxide (DMSO), and frozen for storage in liquid nitrogen. Cell flasks were rinsed and any floating cells were removed after 24 hours of incubation at 37°C with 5% CO_2 at saturating humidity. The remaining adherent cells were expanded in DM. Cells for storage were frozen in 90% FBS and 10% DMSO and held in liquid nitrogen until thawing. The number of passages that occurred prior to cells reaching replicative capacity was recorded.

Results

Stromal cells were successfully isolated from canine umbilical cords. (Figure 2-1). When healthy and viable, the WJMSCs exhibited a typical mesenchymal cell appearance with elongated nuclei and stellate cytoplasm. When reaching their replicative limit, the cells exhibited a round to ovoid appearance and would eventually detach from the culture flask.

Canine WJMSC survived freezing and thawing. The replicative capacity was determined to be between 5 and 10 passages for the cell line used. Both canine WJMSCs and OSA D17 cells survived culture in DM.

The source of the umbilical cord was an English bulldog; however, umbilical cords from other breeds of dogs were also submitted. WJMSCs were also isolated from other dog sources, but not utilized for the remainder of the data reported here.

Discussion

WJMSCs were successfully isolated from a canine umbilical cord. The isolation procedure was fairly easy to perform and yielded a large number of WJMSCs for study; however, the procedure is somewhat time consuming and needs to be performed fairly soon after umbilical cord harvest. The cells isolated appeared to be a homogenous population with a typical mesenchymal cell appearance (Figure 2-1) and survived multiple freeze and thaw cycles with little to no adverse cellular effects observed.

The WJMSCs isolated survived for fewer passages than what has been reported for stromal cells obtained from other sources of WJMSCs as some investigations have indicated that WJMSCs may be expanded for more than 15 passages [36]. There are many possible explanations for the short life span observed with this study.

One possibility is that the DM may not be the ideal culture medium for the canine WJMSCs. Most investigations of human umbilical cord mesenchymal stromal cells have utilized Dulbecco's modified Eagle medium (DMEM) for cell culture [71, 72]. It may be that DMEM, rather than DM, may be the optimal medium for culture of canine WJMSCs. Further

investigations comparing the replicative limit of cells cultured in both mediums should be performed.

Another possibility is that the low replicative limit for this cell line is due to the breed of dog, an English bulldog. English bulldogs typically weigh around 40-50 pounds and are considered to be a medium size breed. These dogs in general have shorter life spans than smaller breed dogs such as Chihuahuas or miniature poodles. It is possible that the differences in the expected life span between breeds may lead to differences in cell survival. Comparing WJMSC isolates from various breeds may show differences in cell viability.

It is also possible that the species, rather than the breed may have an effect on the WJMSC life span. This may be due to an inherent limit on cell survival called the Hayflick limit of canine WJMSCs, which is defined as the number of times a normal cell population will divide before it stops. It is presumed that cells reach their Hayflick limit due to the shortening of telomeres and that a cell's life span can be lengthened via telomere maintenance. Isolation and comparison of WJMSCs from other species, including comparisons of telomere lengths, may prove that an inherent difference between WJMSCs from various species exists.

Lastly, the oxygen tension maintained in the culture incubator may play a role in WJMSC survival. The oxygen saturation in the cell culture incubator is typically set at atmospheric, not physiologic, levels which may lead to cellular hypoxia and a shortened life span. One way to investigate this possibility would be to culture the cells under a low oxygen tension to determine if this truly has an effect on the culture of WJMSCs.

Figures and Tables

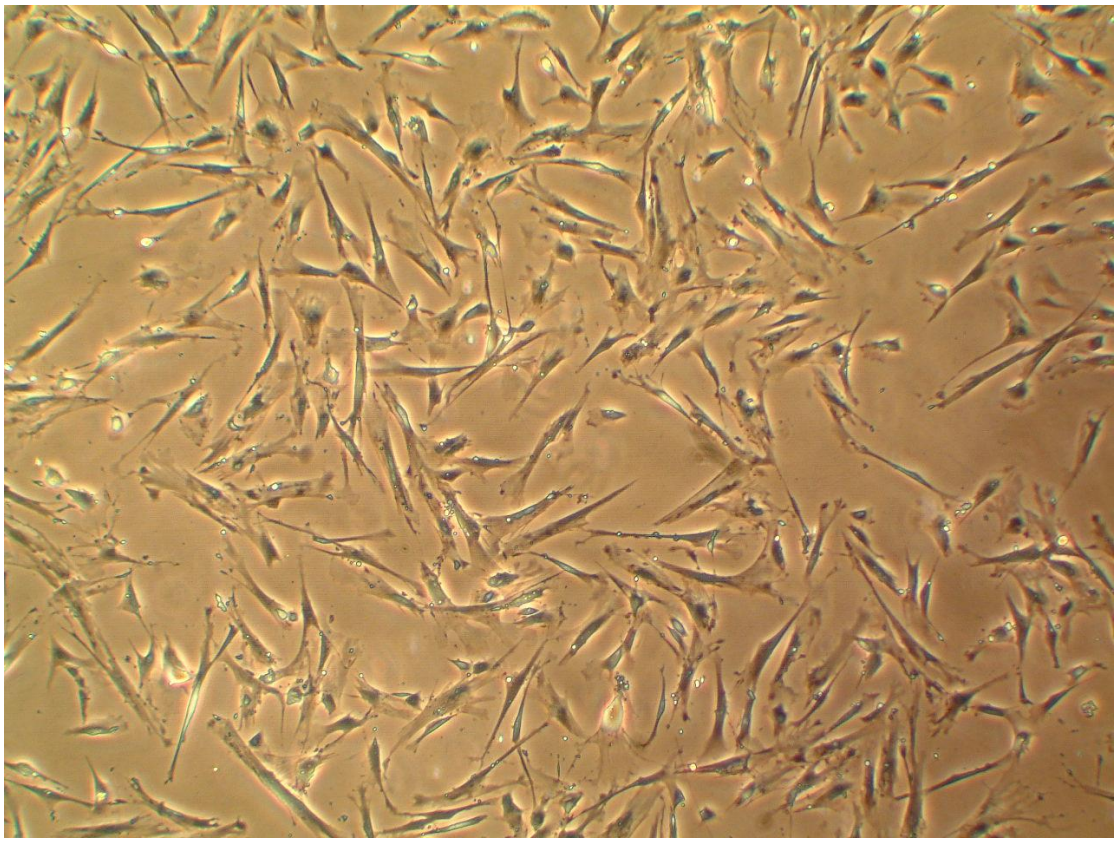

Figure 2-1 Canine Wharton's Jelly Mesenchymal Stromal Cells in Culture at 10x magnification

CHAPTER 3 - Formation of Micellar Nanoparticles Containing Doxorubicin

Hypothesis

Micellar nanoparticles containing doxorubicin can be engineered *in vitro*. Once formed, the micellar nanoparticles will be taken into (loaded in) WJMSCs after combined incubation.

Materials and Methods

Polymeric Micelles

Micelles containing DOX were prepared as previously described, substituting DOX for AmB [69]. Briefly, 1,2-Distearoyls*n*-glycero-3-phosphoethanolamine-*N*-methoxy (polyethylene glycol, M_n=3016 g/mol) (PEG-DSPE)[g], cholesterol[b], and DOX[b] were utilized to formulate the micelles. A round bottom flask was utilized to mix 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 to obtain the desired DOX: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 buffer at a pH of 7.0 and incubated at 50° C for 10 minutes to allow for equilibration. The solution was then filtered through a 0.45 μm polyethersulfone (PES) syringe filter. The concentration of DOX was quantified by using a standard curve for DOX by high performance liquid chromatography (HPLC).

Loading of Polymeric Micelles into Canine WJMSCs

The DOX micelles were provided as a 1mM concentration. Serial dilutions were performed with DM to obtain DOX micelle concentrations of 10μM, 1μM, 0.1μM, 0.01μM, and 0.001μM. Canine WJMSCs were plated at a density of 15,000 cells/well of a 24 well plate. After incubation for 24 hours, the old media was removed from the wells containing WJMSCs and new media containing DOX micelles at concentrations ranging from 10μM to 0.001μM was added. After the addition of the micelles, the WJMSCs were allowed to incubate for 24 hours at which time the old media was removed and fresh DM that did not contain micelles was added. The WJMSCs were then observed under a microscope with a fluorescent filter to confirm DOX fluorescence within the cells and thus micelle uptake.

Results

PEG-DSPE|cholesterol micelles with a narrow particle distribution and containing DOX were successfully formed. Loading of the DOX micelles into WJMSC was confirmed (Figure 3-1).

Discussion

Cellular entry and uptake is required for a drug to achieve therapeutic efficacy. Improving the poor solubility of many anticancer drugs with the use of a NP DDS is one way to improve a drug's therapeutic index. NPs are able to enhance the intracellular concentration of drugs by entering the cells via specific membrane receptors and endocytosis. DOX is a water soluble drug; however, encapsulation within the PMs should provide a therapeutic advantage via reduction of toxicity and increased accumulation in tumor as opposed to normal tissue.

DOX alone is an effective drug for many canine cancers, including OSA. While normal tissue toxicity is a concern, another problem that is commonly observed with DOX is resistance, most commonly mediated through MDR and p-glycoprotein. DOX also lacks the ability to traverse the intact BBB as it is a p-glycoprotein substrate. Using PMs to encapsulate DOX allows the drug to bypass MDR and avoid cellular extrusion via p-glycoprotein [46, 67, 73]. This would allow DOX to remain a viable treatment option for patients that may have developed resistance, such as patients with lymphoma. It may also allow DOX to become a viable treatment option for patients with other cancers, such as brain tumors, because the PM formulation would also allow passage through the BBB.

An as yet unanswered question related to this DOX formulation is what type of toxic side effects can be expected with clinical usage. The multiple toxicities associated with the use of free DOX are well known. The PM formulation reported here will allow the DOX to be released slowly from the carrier molecule, thereby changing the pharmacokinetic parameters of the drug. It is not currently known how these changes will affect the drug's toxicity profile and dosing strategy. *In vitro* studies looking into how and when the DOX is released from the PM and, eventually, *in vivo* pharmacokinetic and toxicity evaluations in live animals will be able to answer these questions.

A pegylated liposomal formulation of DOX, known as Doxil, has been developed to enhance tissue distribution and alter the pharmacokinetic parameters of DOX [32]. The advantages of this NP formulation of DOX include an extended drug half-life and a decreased risk of cardiotoxicity with enhanced efficacy. Doxil has shown efficacy against ovarian cancer [60, 74], Kaposi's sarcoma [59], and multiple myeloma [61, 75] in humans. In veterinary medicine, Doxil has been investigated for use in dogs with hemangiosarcoma [62] and cats with

soft tissue sarcomas [63, 64]. While some efficacy has been observed in the dogs and cats studied, the use of Doxil in veterinary medicine is limited by cost, availability, and toxicity. The dose limiting toxicity in both humans and animals is a syndrome called palmar plantar erythrodysesthesia (PPES), or hand-foot syndrome. PPES is a progressive accumulation of painful nodules and erythematous desquamation on the palms of the hands and soles of the feet in humans and areas of skin contact in dogs including the axilla, inguinal region, and skin surrounding the foot pads [32, 76]. Pyridoxine has been shown to be effective at delaying the onset and severity of PPES in dogs [76]; however, if PPES develops, it most likely leads to discontinuation of Doxil therapy. Other possible toxicities associated with the use of Doxil include anaphylactic-like reactions, gastrointestinal toxicity, and azotemia and nephrotoxicity in cats [63, 76].

The PM formulation of DOX reported here will hopefully not exhibit the adverse effects observed with the use of Doxil. Much more research is needed to determine how the toxicity profiles between free DOX, DOX micelles, and Doxil differ, most importantly in relation to cumulative cardiotoxicity, nephrotoxicity, and PPES. The lack of toxicity information is a problem with use of NPs in general. While they are ideal to modulate and overcome drug solubility and stability issues, exposure to certain NPs may pose risks to normal tissue. The fate of both biodegradable and non-biodegradable NPs and their constituents are also unknown. These unknowns become serious when considering the intravenous injection of NPs as improper targeting may occur [48]. Loco-regional injection may be considered a viable substitute; however, improvement of NP tumor targeting utilizing cells, such as WJMSCs, that possess disease homing capabilities is an attractive alternative.

Figures and Tables

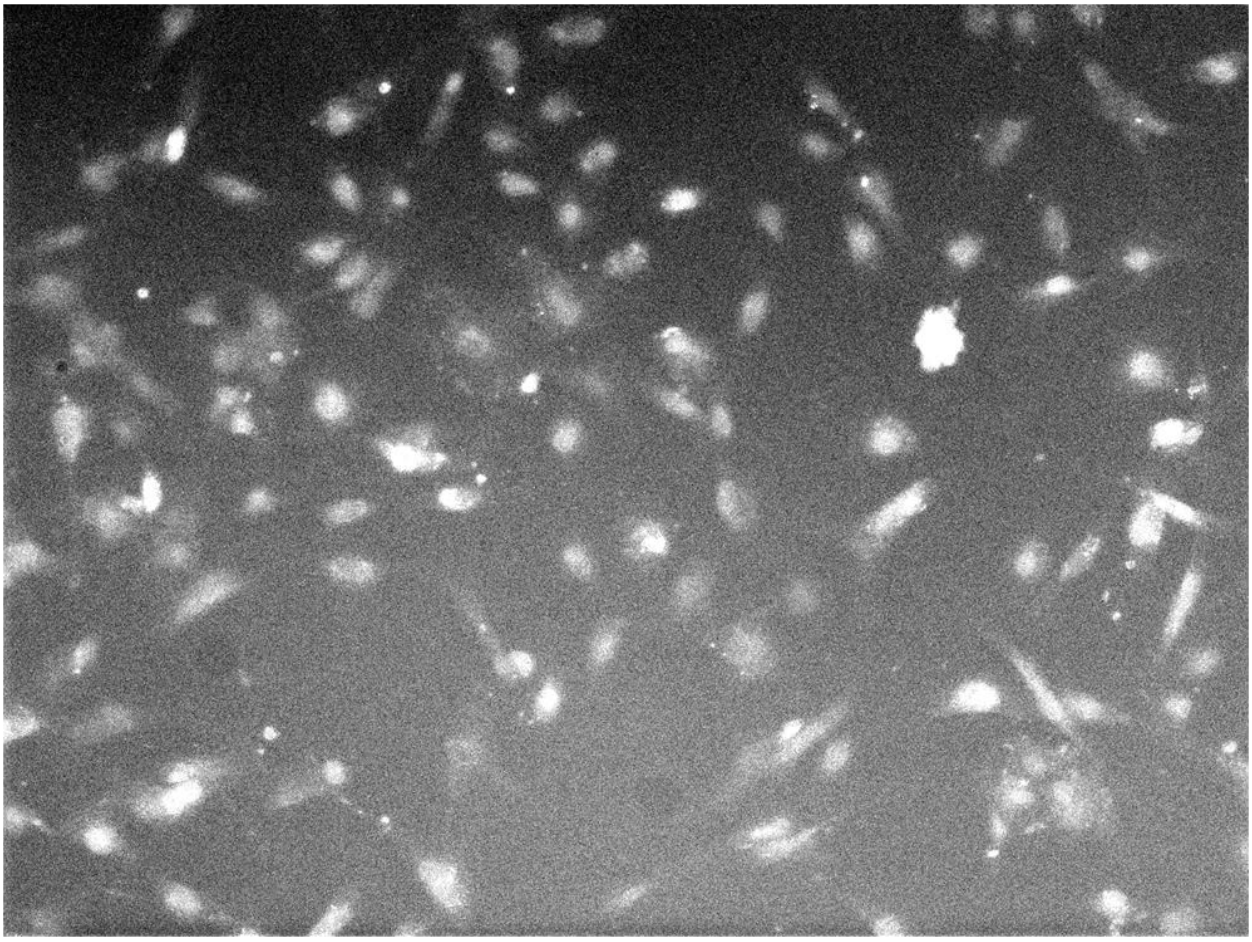

Figure 3-1 WJMSCs containing DOX Micelles at 10x magnification

CHAPTER 4 - Changes Observed in Osteosarcoma D17 Cell Viability after Incubation with Micellar Nanoparticles Containing Doxorubicin

Hypothesis

Micellar nanoparticles containing doxorubicin will negatively affect OSA D17 cell viability *in vitro*.

Materials and Methods

OSA D17 cells were obtained from the American Type Culture Collection (ATCC)[h]. These cells represent an OSA cell line obtained from a pulmonary metastatic lesion in a poodle. The cells were housed in DM and incubated at 37°C with 5% CO_2 at saturating humidity. Cells for storage were frozen in 90% FBS and 10% DMSO and held in liquid nitrogen until thawing.

Effects of the DOX micelles on OSA D17 cells were determined by incubating DOX micelles with OSA D17 cells. As in the previous experiment, the DOX micelles were provided as a 1mM concentration and serial dilutions were performed with DM to obtain concentrations of 10μM, 1μM, 0.1μM, 0.01μM, and 0.001μM.

OSA D17 cells were plated at a density of 1700 cells/well in a 96 well plate. After 24 hours of incubation in DM, old media was removed and new media containing DOX micelles at concentrations ranging from 10μM to 0.001μM was added. OSA D17 cells without DOX micelles served as a negative control and OSA cells incubated with free DOX at a concentration of 10μM served as a positive control.

At time points of 24, 48, 72, and 96 hours post DOX micelle addition, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT)[i] was performed, per the manufacturer's protocol, to determine OSA D17 cell viability and proliferation. The results were plotted as a percent of the control population. Each variable was evaluated in triplicate during three separate experiments. Results were then averaged at each time point.

Results

Micellar NPs containing DOX decreased OSA D17 cell viability. Growth inhibition of greater than 80% was observed at 96 hours with the free DOX and the 10μM and 1μM DOX micelles (Figure 4-1). Growth inhibition of approximately 65% was observed at 96 hours with the 0.1μM DOX micelles (Figure 4-1). Growth inhibition was similar between free DOX and both the 10μM and 1μM DOX micelles at 72 and 96 hours. The maximum growth inhibition at 24, 48, and 72 hours was observed with the 10μM DOX micelles and with both the 10μM and 1μM DOX micelles at 96 hours.

Discussion

DOX micelles negatively affect OSA D17 cell viability *in vitro.* The greatest effect was observed with cells incubated with 10μM and 1μM DOX micelles at 96 hours; however, the 10 μM free DOX exhibited similar results at the 96 hour time point. Even a lower DOX micelle concentration of 0.1 μM showed a substantial negative effect at 96 hours. An interesting finding was that both the 10μM and 1μM concentrations of DOX micelles exhibited greater cytotoxicity than the free DOX at the 72 and 96 hour time points. These results indicate that encapsulation into PMs increases rather than decreases the cytotoxicity of DOX.

Even though similar cytotoxicity was observed between free DOX and higher concentrations of DOX micelles, the use of PMs as a DDS for DOX may provide multiple treatment advantages. One of the main advantages of housing DOX in PMs would be to allow for a more controlled release of the drug. One of the most troublesome side effects of DOX usage is a cumulative cardiotoxicity that has been observed in both human and canine patients. There are ways to lessen the risk of cardiotoxicity in humans including screening patients for preexisting heart disease, treating hypertension, and minimizing cardiac irradiation [32]. While many canine patients receiving DOX are screened for underlying heart disease with echocardiograms, it is unknown if hypertension and cardiac irradiation increase the risk of cardiac toxicity. It has been widely reported that the peak drug concentration of DOX is a major contributor to cardiac toxicity risk while the antitumor activity is a function of the area under the curve (AUC) [32]. Using dilute formulations of DOX and giving the infusion slowly, typically over 30 minutes in dogs, are two ways that these differences can be exploited. The micelles used for the study reported here are identical to those reported by Vakil and Kwon in 2008 [69] and as such should exhibit the same stable incorporation and slow release of drug as was observed in their study. These characteristics would allow for a more controlled, sustained release of the DOX from the micelles, leading to a longer AUC and lower peak drug concentration. If found to be safe *in vivo*, these DOX micelles may provide another way to help decrease the risk of cumulative cardiotoxicity.

The micellar formulation of DOX would also allow for a longer circulation time for the drug via avoidance of the RES. As discussed previously, the RES removes drugs from circulation via opsonization and phagocytosis [51]. Longer circulation times would allow for more drug to be delivered to the target tissue to cause the desired therapeutic effect. One

possible problem with a longer circulation time is that the DOX may accumulate in areas other than the target tissue. Utilizing DOX micelles in combination with another tissue targeting component may decrease the possibility that inappropriate accumulation occurs.

The results of this experiment indicate that DOX micelles may be a good substitute for free DOX as similar cytotoxic effects were observed. Further study should be performed to see if this observation remains true *in vivo*.

Figures and Tables

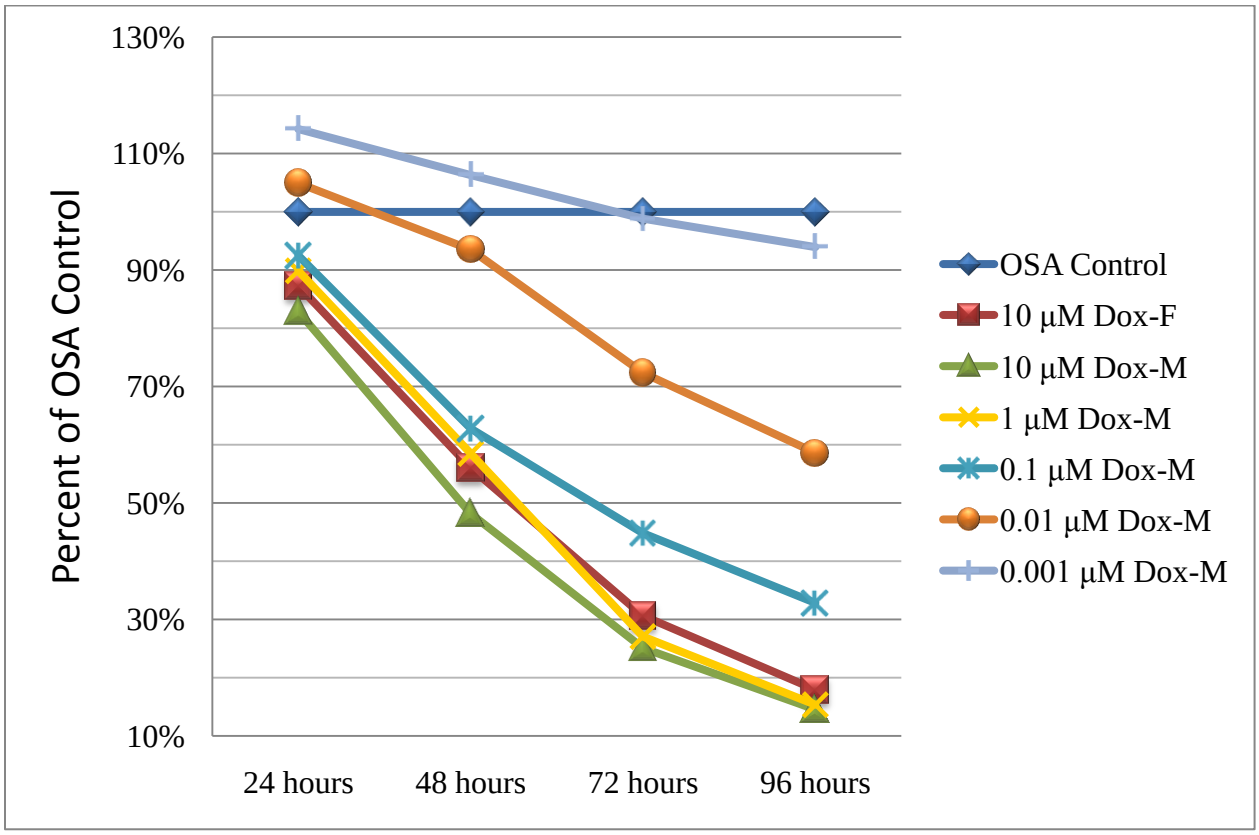

Figure 4-1 OSA D17 Cell Viability Following 24 Hour Incubation with DOX micelles

CHAPTER 5 - Determination of Osteosarcoma D17 Cell Viability after Incubation with Conditioned Media from Wharton's Jelly Mesenchymal Stromal Cells Loaded with Micellar Nanoparticles Containing Doxorubicin

Hypothesis

Canine WJMSCs loaded with micellar nanoparticles containing doxorubicin will negatively affect canine OSA D17 cell viability *in vitro*.

Materials and Methods

Conditioned Media

Effects of WJMSCs containing NPs 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 micelles. DOX micelles were provided as a 1mM concentration and serial dilutions were performed with DM to obtain concentrations of 10μM, 1μM, 0.1μM, 0.01μM, and 0.001μM.

To obtain the CM, canine WJMSC were plated at a density of 15,000 cells/well of a 24 well plate (Day 1). After 24 hours of incubation the old media was removed and new DM containing DOX micelles at concentrations of 10μM to 0.001μM was added (Day 2). After 24 hours of incubation, the WJMSCs containing DOX micelles were viewed with a fluorescent microscope to confirm the presence of doxorubicin within the WJMSCs. After confirmation of loading, the old media containing DOX micelles was removed and new DM without micelles was added (Day 3). Once DOX micelle loading into the WJMSCs was confirmed, OSA D17

cells were plated at a density of 1700 cells/well of a 96 well plate. Plating occurred on the same day as the media change for the WJMSCs (Day 3). Both the WJMSC and OSA D17 cell lines were allowed to incubate for 24 hours, then examined to determine if the cells were healthy and increasing in confluency (Day 4). If the cells were deemed healthy, they were allowed to incubate for another 24 hours at which time the media, now known as the CM, was removed from the culture wells of the WJMSCs containing DOX micelles and transferred to the culture wells containing OSA D17 cells (Day 5). Wells containing only OSA D17 cells with fresh DM, OSA D17 cells with free DOX at a concentration of 10μM, and OSA D17 cells with CM from unloaded WJMSC served as controls.

At time points of 24, 48, 72, and 96 hours post DOX micelle addition, an MTT assay[i] was performed, per the manufacturer's protocol, to determine OSA D17 cell viability and proliferation (Days 6-10). The results were plotted as a percent of the control population. Each variable was evaluated in triplicate during three separate experiments. Results were then averaged at each time point.

The methods for this experiment are summarized in table 5-1.

Polymeric Micelle Effects on Canine WJMSCs

Upon transfer of conditioned media, the WJMSC were harvested with trypsin and trypan blue stain was added. The canine WJMSC were then counted with a hemocytometer to determine if the addition of the NPs affected the number of live versus dead WJMSCs. Each variable was evaluated in triplicate during 3 separate experiments. The cell counts from all experiments were then averaged and graphed.

Statistical Evaluation

Commercially available statistical software[j] was utilized to determine what variables exerted significant changes in OSA D17 cell viability. A mixed model repeated measures analysis using Kenward-Roger degrees of freedom approximation methods was performed using the SAS/MIXED procedure to determine if significant differences existed between each variable at each time point. A heterogenous first-order autoregressive correlation structure was selected for the model by optimizing the fit statistics. Pairwise differences were examined at each time point and a heterogeneous first-order autoregressive correlation structure was selected for the model by optimizing the fit statistics.

Results

Wharton's jelly mesenchymal stromal cell numbers decreased in a dose dependent manner following incubation with DOX micelles (Figure 5-1). The change in WJMSC number was not due to an increase in the number or percentage of dead cells (Table 5-2).

Comparing all variables to the control population, OSA D17 cell viability was decreased following incubation with CM obtained from canine WJMSCs loaded with DOX micelles. Significant reductions were observed with 10 µM free DOX at 48, 72, and 96 hours ($p < 0.0001$). Significant reductions were also observed with the 10 uM DOX micelle concentration at 48 hours ($p < 0.005$) and also at 72 and 96 hours ($p < 0.001$). The 1 uM DOX micelle concentration also showed significant reduction in OSA D17 viability at 72 ($p < 0.005$) and 96 ($p < 0.001$) hours. (Figure 5-2)

Significant inter-variable differences were also observed. At 48, 72, and 96 hours, the 10 µM free DOX was significantly different than the unloaded WJMSCs, the 0.1 µM DOX

micelles, the 0.01 µM DOX micelles and the 0.001 µM DOX micelles ($p < 0.0001$ for all comparisons). A significant difference between the 10 µM free DOX and 1 µM DOX micelles was found at 96 hours ($p < 0.0001$). The 10 µM DOX micelles were significantly different than the unloaded WJMSCs and the 0.1 µM DOX micelles at 72 and 96 hours ($p < 0.0001$). The 10 µM DOX micelles were also significantly different than the 0.01 µM and the 0.001 µM DOX micelles at 48 hours ($p=0.0268$ and $p=0.0089$ respectively), and 72 and 96 hours ($p < 0.0001$). Even the 1 µM DOX micelles were significantly different than the unloaded WJMSCs at 72 ($p = 0.0027$) and 96 hours ($p < 0.0001$), the 0.1 µM DOX micelles at 72 ($p = 0.0008$) and 96 hours ($p < 0.0001$), the 0.01 µM DOX micelles at 72 ($p = 0.0008$) and 96 hours ($p < 0.0001$), and the 0.001 µM DOX micelles at 72 ($p = 0.0024$) and 96 hours ($p < 0.0001$). See table 5-3 for a summary of the inter-variable significance.

Discussion

The results reported here provide evidence that the CM from WJMSCs containing DOX micelles have cytotoxic effects on OSA D17 cells *in vitro*. The decrease in OSA D17 cell viability observed in this study was similar to that observed with DOX micelles alone. These findings indicate that loading the NPs into the WJMSCs does not diminish cytotoxicity. In fact, in an *in vivo* situation, the cytotoxic effects may actually be enhanced with the combined use of canine WJMSCs and DOX micelles. This is supported by the cytotoxicity observed in the OSA D17 cells that were exposed to CM from unloaded WJMSCs. These results are promising and indicate the need for further study into DOX micelle and WJMSCs usefulness in treating canine OSA.

The CM from the WJMSCs containing the two highest concentrations of DOX micelles, 10 µM and 1 µM, exhibited similar cytotoxic effects when compared to the free DOX. The only significant difference among these variables was found at the 96 hour time point between the 1 µM DOX micelles and the 10 µM free DOX. Further, the 10 µM and 1 µM DOX showed some significant differences when compared to the other micelle concentrations studied. These findings indicate that further study should focus on determining if the effects observed with the 10 µM and 1 µM DOX micelles *in vitro* remain true *in vivo*.

When comparing the overall number and percentages of dead WJMSC cells, there appears to be an anti-proliferative, rather than a cytotoxic effect. When injected systemically, the WJMSCs require approximately 24 hours to localize to the lung tissue. In an *in vivo* situation, it should not matter that the WJMSCs are not proliferating. As long as they remain intact and do not die, they should be able to reach their therapeutic target and deliver the NP payload to the intended target. *In vitro* co-culture experiments are performed to study interactions between cells. There are many ways to perform co-culture experiments including direct mixture of cell lines or the use of Transwell® culture plates to keep the cell lines separate. The CM approach utilized in this study was chosen to more accurately simulate the *in vivo* EPR effect [54]. It is possible that direct contact between WJMSCs and OSA D17 cells, similar to what would occur in an *in vivo* situation in which the WJMSCs deliver the PMs directly to the tumor, may produce even greater cytotoxicity. Further *in vitro* work utilizing more direct methods of co-culture is suggested to study this interaction.

While the possibility of utilizing WJMSCs and DOX micelles for canine OSA is exciting, this targeted form of therapy may also provide new treatment options for patients with previously inaccessible disease and patients with cancer that is resistant to chemotherapy. For example,

there are not many options available for veterinary patients with intracranial neoplasia as surgical removal of tumors is rarely performed, the optimal chemotherapy agent is not currently known, and drug delivery to the site of disease is difficult. Radiation therapy is an option; however, many owners cannot afford it, are unable or unwilling to travel to a radiation facility, or are reluctant to deal with radiation side effects. Other cancers in dogs and cats, such as those of the pancreas, prostate, and heart, are also therapeutic challenges as they cannot be removed and truly effective chemotherapies are not known. Even though these tumors are considered rare, if a targeted approach could be utilized, we may be able to develop more effective treatments and expect longer survival times.

The OSA D17 cytotoxicity observed in these studies indicates that a combination of WJMSCs and DOX micelles may be an effective therapy for canine OSA *in vivo*. Further study should be performed to determine if WJMSCs will actually home to a disease target and deliver a therapeutic payload. There are many ways that this may be determined; however, the first steps in proving this theory should be to determine if the DOX micelles and/or the WJMSCs cause adverse effects in animals. A mouse should be the initial study subject to determine if adverse effects of other NPs, including pulmonary toxicity and platelet aggregation, are observed after systemic injection of DOX micelles. Initial investigations of DOX micelle toxicity should utilize the 10 μM concentration as this induced cytotoxicity at a similar level to the free DOX. It is unknown what side effects occur after systemic injection of WJMSCs, therefore, this should also be investigated. Once both the NP and the delivery vehicle are found to be safe, the investigations should be moved into normal dogs and then into veterinary oncology patients, as diseased animals do not always exhibit the same toxicity profiles as their normal counterparts.

After determining the safety of DOX micelles and WJMSCs, the next step should be to determine if the canine WJMSCs can home to specific tissues, as has been shown with WJMSCs of other species [35]. One way to track the WJMSCs after systemic injection is to engineer the WJMSCs to synthesize the luciferase gene. A bioluminescence imaging system would then be used to track the cells over time. Another way would be to develop an antibody that binds to the WJMSCs that could be used to detect their presence in tissue. The antibody could then be utilized for immunohistochemistry. Once homing ability has been determined, the next step would be to confirm that the DOX micelles remain within the WJMSCs during travel and are delivered to the target tissue to cause a therapeutic effect. Active targeting in which targeting ligands are attached to the WJMSCs should also be investigated.

The combined, targeted approach of WJMSCs and PMs is a fairly new approach in nanotechnology. Nevertheless, newer applications of this combination are already on the horizon with researchers beginning to examine if NPs can be used as a "theranostic." The structure of PMs is very unique and may allow the incorporation of both an imaging agent and a therapeutic agent. Utilizing the WJMSCs to deliver these multi-functional PMs may allow clinicians to both diagnose and treat disease while also offering a way to monitor response to therapy [53].

One limitation of the study is that all assumptions of future utilization of this novel therapeutic approach are currently based on *in vitro* work. While these results are exciting, it is important that they be confirmed with *in vitro* studies. Only then can the full potential of a targeted approach utilizing WJMSCs and NPs be discovered.

Conclusions

The overall conclusion from these data indicate that DOX micelles are cytotoxic to OSA D17 cells and that cytotoxicity may be enhanced with the combined use of canine WJMSCs and DOX micelles. While much work still needs to be done, these data indicate that WJMSCs and DOX micelles may provide a clinically relevant therapeutic option for veterinary oncology in the future. If proven clinically relevant for dogs with OSA, it is possible that one day this novel therapy may be translated into human oncology.

The potential applications for this type of therapy are virtually endless when one considers that just about any anticancer drug can be encapsulated in a NP and delivered to virtually any type of tumor. The full extent of disease may be detected, chemotherapy resistance may be eliminated, and more effective treatments may be developed. These changes would revolutionize the field of oncology and greatly alter how we diagnose and treat cancer.

Figures and Tables

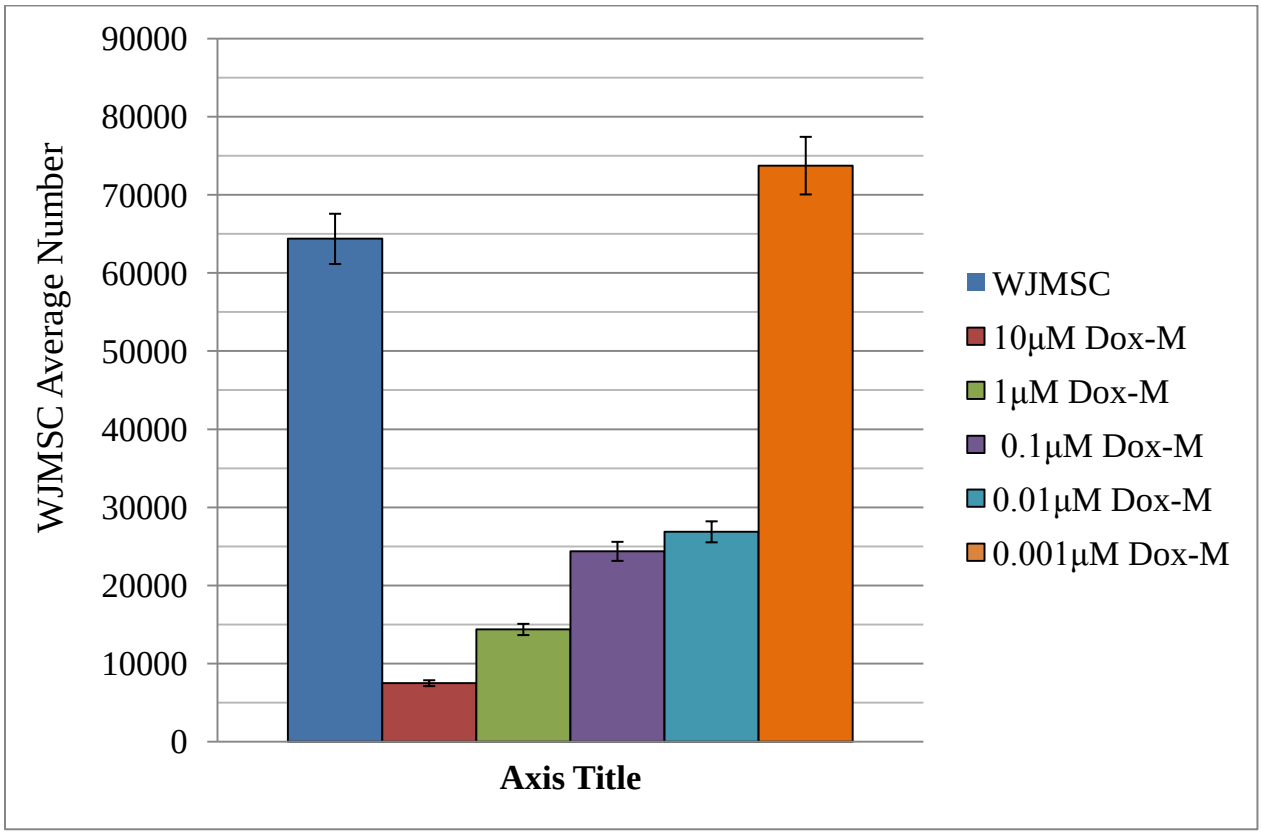

Figure 5-1 Changes in Live WJMSC Counts after 24 Hour Incubation with Micellar NPs Containing DOX. Error bars represent a 5% value.

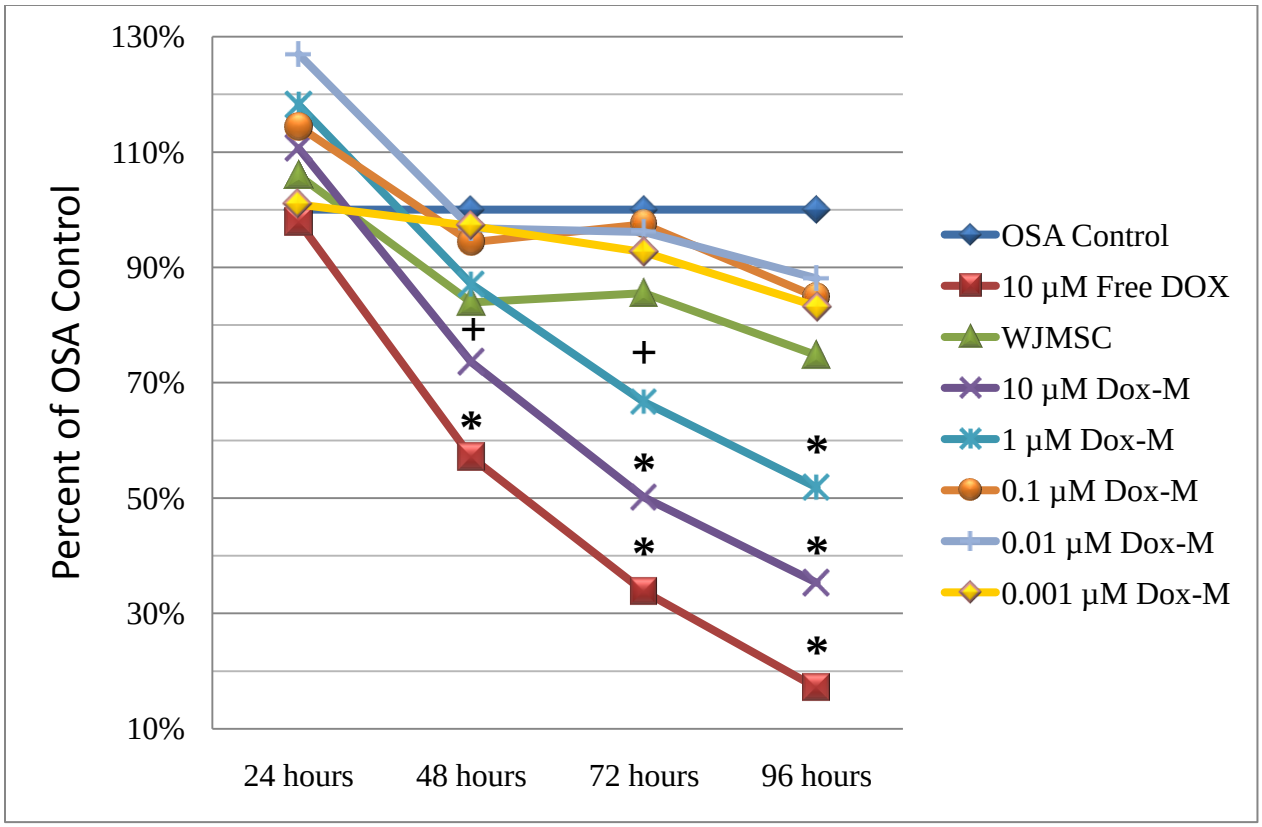

Figure 5-2 Changes in Canine OSA D17 Cell Viability after Incubation with CM from WJMSCs Loaded with DOX Micelles. The CM utilized was obtained from wells containing WJMSCs that were loaded with the listed concentrations of DOX micelles.

* Indicates statistically significant values with a p-value less than 0.0001

+ Indicates statistically significant values with a p-value less than 0.005

Table 5-1 Summary of Conditioned Media Experiment Methods

Day	Action
1	• Plate canine WJMSCs in 24 well plate at a density of 15,000 cells/well
2	• Perform serial dilutions of DOX micelles • Add DOX micelles to WJMSCs
3	• Examine WJMSCs to confirm loading of DOX micelles • Remove old media containing micelles from WJMSCs and add fresh DM • Plate OSA cells in a 96 well plate at a density of 1700 cells/well
4	• Examine plates containing WJMSCs and OSA D17 cells for viability
5	• Remove old media from OSA D17 cells • Transfer CM from loaded and unloaded WJMSCs to OSA D17 cells • Perform trypan stain and count WJMSCs
6	• Begin MTT assay on 24 hour OSA D17 wells
7	• Complete MTT assay on 24 hour OSA D17 wells • Begin MTT assay on 48 hours OSA D17 wells
8	• Complete MTT assay on 48 hour OSA D17 wells • Begin MTT assay on 72 hour OSA D17 wells
9	• Complete MTT assay on 72 hour OSA D17 wells • Begin MTT assay on 96 hour OSA D17 wells
10	• Complete MTT assay on 96 hour OSA D17 wells
11	• Read absorbance of plate containing MTT wells with spectrophotometer and record results

Table 5-2 Percent of Dead WJMSCs Post 24 Hour Incubation with DOX Micelles

Variable	Percent Dead Cells
Unloaded WJMSCs	7.28%
10 µM DOX-M	13.89%
1 µM DOX-M	6.78%
0.1 µM DOX-M	4.65%
0.01 µM DOX-M	7.55%
0.001 µM DOX-M	2.49%

Table 5-3 Inter-variable Significance

	Comparison Variable	**Time Points** (in hours)	**p value**
10 µM free DOX	Unloaded WJMSCs	48, 72, 96	< 0.0001
	1 µM DOX micelles	96	< 0.0001
	0.1 µM DOX micelles	48, 72, 96	< 0.0001
	0.01 µM DOX micelles	48, 72, 96	< 0.0001
	0.001 µM DOX micelles	48, 72, 96	< 0.0001
10 µM DOX micelles	Unloaded WJMSCs	72, 96	< 0.0001
	0.1 µM DOX micelles	72, 96	< 0.0001
	0.01 µM DOX micelles	48 72, 96	0.0268 < 0.0001
	0.001 µM DOX micelles	48 72, 96	0.0089 < 0.0001
1 µM DOX micelles	Unloaded WJMSCs	72 96	0.0027 < 0.0001
	0.1 µM DOX micelles	72 96	0.0008 < 0.0001
	0.01 µM DOX micelles	72 96	0.0005 < 0.0001
	0.001 µM DOX micelles	72 96	0.0024 < 0.0001

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Appendix A - Vendors

a – Invitrogen, Carlsbad, CA

b – Sigma, St. Louis, MO

c – Atlanta Biologicals, Atlanta, GA

d – R & D Systems, Minneapolis, MN

e – Fisher Scientific, Rockford, IL

f – Seward Ltd., Thetford, Norfolk, UK

g – Avanti Polar Lipids, Alabaster, AL

h – American Type Culture Collection, Manassas, VA

i – Roche Applied Science, Indianapolis, IN

j – SAS, SAS Institute Inc., Cary, NC