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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 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 $CaCl_2$: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% CO_2. 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% CO_2. 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-Distearoylsn-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 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, 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

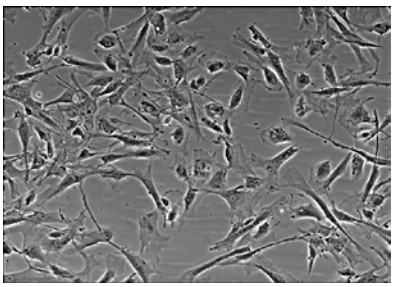

Figure 1A: Bright field view of canine WJMSC at 10X power.

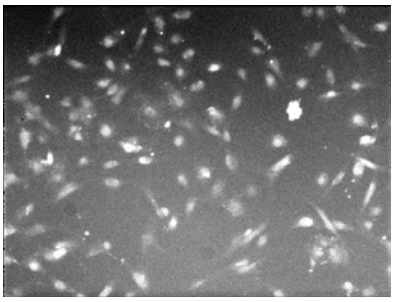

Figure 1B: Same microscopic field as in Figure 1A, viewed with a fluorescent filter (TRITC filter, excitation BP 545/25, emission BP 605/70) to detect the presence of DOX-M within the WJMSC.

experiments. The results were averaged at each time point and plotted as percent of the control population.

Upon transfer of the CM, the WJMSC were harvested with trypsin and trypan blue stain was added. The WJMSC were counted with a hemocytometer to determine if the addition of the DOX-M affected WJMSC number or viability.

Statistical analysis: Data were analyzed as a mixed model with repeated measures using the SAS/GLIMMIX procedure (SAS software) with Kenward-Roger degrees of freedom approximation methods. 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. A value of $p \leq 0.05$ was considered significant.

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.

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,

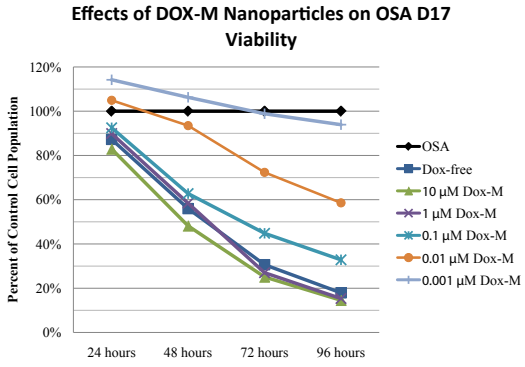

Figure 2: Decreases in OSA D17 cell viability were observed with the addition of DOX-M. Significant differences were found when comparing the OSA control population to OSA cells incubated with 10 μM free DOX, 10 μM DOX-M, and the 1 μM DOX-M at 48 ($p<0.05$), 72 ($p<0.001$), and 96 hours ($p<0.0001$). Significant differences were also observed when comparing the control OSA population to OSA cells incubated with 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).

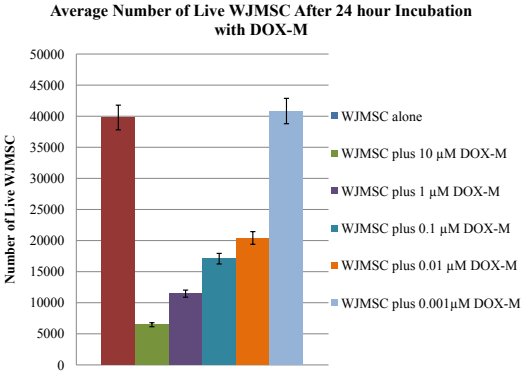

Figure 3: Variability of canine WJMSC associated with the addition of DOX-M nanoparticles. Counts were taken after 24 hour incubation with DOX-M. Live canine WJMSC numbers decreased in a dose dependent manner relative to DOX-M concentration. Error bars represent a 5% value

Variable	Percent Dead WJMSC
Unloaded WJMSC	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 1: Percent of dead canine WJMSC post 24 hour incubation with DOX-M.

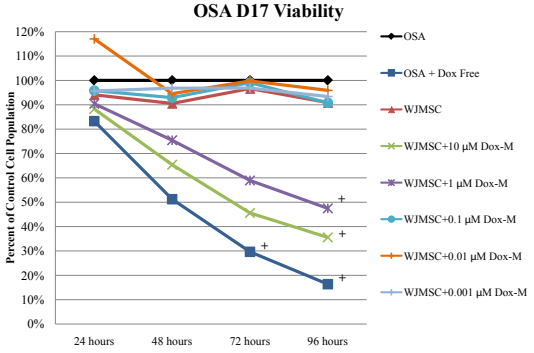

Figure 4: Changes in canine OSA D17 viability after incubation with CM from WJMSC loaded with DOX-M. + indicates a significant difference between the test variable compared to the control population with a p-value ≤ 0.05. Significant differences were found when comparing the 10 μM free DOX at 72 hours to the control OSA population (p=0.0506). Significant differences were also observed between the control OSA population and the 10 μM free DOX, the 10 μM DOX-M, and the 1 μM DOX-M at 96 hours (p=0.002, 0.0037, and 0.0222 respectively).

0.1 μM DOX-M, and 0.01 μM DOX-M with all variables having a p value of <0.0001.

Effect of DOX-M on WJMSC number

WJMSC numbers decreased in a dose dependent manner following incubation with micelles containing DOX (Figure 3).The number and percent of dead WJMSC, however, did not increase with increasing concentrations of DOX-M, indicating an anti-proliferative rather than cytotoxic effect (Table1).

Conditioned media experiments

Comparing all variables to the control population, OSA D17 cell viability was decreased following incubation with CM obtained from canine WJMSC loaded with DOX-M. Significant reductions in OSA D17 cell viability were observed with 10 μM free DOX at 72, and 96 hours (p=0.0506 and 0.0002 respectively). Significant reductions were also observed with the CM from WJMSC loaded with 10 μM DOX-M at 96 hours (p=0.0037) and the CM from WJMSC loaded with 1 μM DOX-M at 96 hours (p=0.0222) (Figure 4).

Discussion

The results of this study show that WJMSC can be isolated from canine umbilical cords and maintained in culture. A large number of WJMSC were obtained and the cells appeared to be a homogenous population, indicating that canine umbilical cords are a good source of WJMSC.

At the time of manuscript preparation, the authors were unaware of similar studies being performed with cells obtained from canine Wharton's jelly; however, new data has been released characterizing cells similar to those presented here [23]. While the cells in this study are classified as stromal cells, it is likely that they are analogous to the canine Wharton'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.

The results reported here provide evidence that the CM from 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

may provide a clinically relevant therapeutic option for OSA in canine and human patients in the future.

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References

1. Withrow SJ, Vail DM (2007) Withrow and MacEwen's Small Animal Clinical Oncology.(4edn), Elsevier: St. Louis, MO.
2. Berg J, Weinstein MJ, Schelling SH, Rand WM (1992) Treatment of dogs with osteosarcoma by administration of cisplatin after amputation or limb-sparing surgery: 22 cases (1987-1990). J Am Vet Med Assoc 200: 2005-2008.
3. Thompson JP, Fugent MJ (1992) Evaluation of survival times after limb amputation, with and without subsequent administration of cisplatin, for treatment of appendicular osteosarcoma in dogs: 30 cases (1979-1990). J Am Vet Med Assoc 200: 531-533.
4. Kraegel SA, Madewell BR, Simonson E, Gregory CR (1991) Osteogenic sarcoma and cisplatin chemotherapy in dogs: 16 cases (1986-1989). J Am Vet Med Assoc 199: 1057-1059.
5. Straw RC, Withrow SJ, Richter SL, Powers BE, Klein MK, et al. (1991) Amputation and cisplatin for treatment of canine osteosarcoma. J Vet Intern Med 5: 205-210.
6. Shapiro W, Fossum TW, Kitchell BE, Couto CG, Theilen GH (1988) Use of cisplatin for treatment of appendicular osteosarcoma in dogs. J Am Vet Med Assoc 192: 507-511.
7. Phillips B, Powers BE, Dernell WS, Straw RC, Khanna C, et al. (2009) Use of single-agent carboplatin as adjuvant or neoadjuvant therapy in conjunction with amputation for appendicular osteosarcoma in dogs. J Am Anim Hosp Assoc 45: 33-38.
8. Bergman PJ, MacEwen EG, Kurzman ID, Henry CJ, Hammer AS, et al. (1996) Amputation and carboplatin for treatment of dogs with osteosarcoma: 48 cases (1991 to 1993). J Vet Intern Med 10: 76-81.
9. Berg J, Weinstein MJ, Springfield DS, Rand WM (1995) Results of surgery and doxorubicin chemotherapy in dogs with osteosarcoma. J Am Vet Med Assoc 206: 1555-1560.
10. Bacon NJ, Ehrhart NP, Dernell WS, Lafferty M, Withrow SJ (2008) Use of alternating administration of carboplatin and doxorubicin in dogs with microscopic metastases after amputation for appendicular osteosarcoma: 50 cases (1999-2006). J Am Vet Med Assoc 232: 1504-1510.
11. Kent MS, Strom A, London CA, Seguin B (2004) Alternating carboplatin and doxorubicin as adjunctive chemotherapy to amputation or limb-sparing surgery in the treatment of appendicular osteosarcoma in dogs. J Vet Intern Med 18: 540-544.
12. Bailey D, Erb H, Williams L, Ruslander D, Hauck M (2003) Carboplatin and doxorubicin combination chemotherapy for the treatment of appendicular osteosarcoma in the dog. J Vet Intern Med 17: 199-205.
13. Chun R, Kurzman ID, Couto CG, Klausner J, Henry C, et al. (2000) Cisplatin and doxorubicin combination chemotherapy for the treatment of canine osteosarcoma: a pilot study. J Vet Intern Med 14: 495-498.
14. Mauldin GN, Matus RE, Withrow SJ, Patnaik AK (1988) Canine osteosarcoma. Treatment by amputation versus amputation and adjuvant chemotherapy using doxorubicin and cisplatin. J Vet Intern Med 2: 177-180.
15. Withrow SJ, Vail DM (2007) Cancer Chemotherapy: Withrow and MacEwen's Small Animal Clinical Oncolog .(4thedn), Elsevier: St LouisMO.
16. Ogilvie GK, Richardson RC, Curtis CR, Withrow SJ, Reynolds HA, et al. (1989) Acute and short-term toxicoses associated with the administration of doxorubicin to dogs with malignant tumors. J Am Vet Med Assoc 195: 1584-1587.
17. Rachakatla RS, Balivada S, Seo GM, Myers CB, Wang H, et al. (2010) Attenuation of Mouse Melanoma by A/C Magnetic Field after Delivery of Bi-Magnetic Nanoparticles by Neural Progenitor Cells. ACS Nano 4: 7093-7104.
18. Rachakatla RS, Marini F, Weiss ML, Tamura M, and Troyer D (2007) Development of human umbilical cord matrix stem cell-based gene therapy for experimental lung tumors. Cancer Gene Ther 14: 828-835.
19. Ayuzawa R, Doi C, Rachakatla RS, Pyle MM, Maurya DK, et al. (2009) Naïve human umbilical cord matrix derived stem cells significantly attenuate growth of human breast cancer cells *in vitro* and *in vivo*. Cancer Lett 280: 31-37.
20. Ganta C, Chiyo D, Ayuzawa R, Rachakatla R, Pyle M, et al. (2009) Rat umbilical cord stem cells completely abolish rat mammary carcinomas with no evidence of metastasis or recurrence 100 days post-tumor cell inoculation. Cancer Res 69: 1815-1820.
21. Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, et al. (2005) Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol 33: 1402-1416.
22. Vakil R, Kwon GS (2008) Effect of cholesterol on the release of amphotericin B from PEG-phospholipid micelles. Mol Pharm 5: 98-104.
23. Seo MS, Park SB, Kang KS (2012) Isolation and characterization of canine Wharton's jelly-derived mesenchymal stem cells. Cell Transplant.
24. Rachakatla RS, Troyer D (2009) Wharton's jelly stromal cells as potential delivery vehicles for cancer therapeutics. Future Oncol 5: 1237-1244.
25. Tamura M, Kawabata A, Ohta N, Uppalapati L, Becker KG, et al. (2011) Wharton's Jelly Stem Cells as Agents for Cancer Therapy. Open Tissue Eng and Regen Med J 4: 39-47.
26. Mei SH, McCarter SD, Deng Y, Parker CH, Liles WC, et al. (2007) Prevention of LPS-induced acute lung injury in mice by mesenchymal stem cells overexpressing angiopoietin 1. PLoS Med 4: e269.