Journal of
Stem Cell Research & Therapy

Rici et al., J Stem Cell Res Ther 2013, 3:3
DOI: 10.4172/2157-7633.1000143

Research Article Open Access

Modulation of Angiogenesis and Immune Response in Canine Osteosarcoma by BMP-2 and Mesenchymal Stem Cells

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Abstract

Osteosarcoma is the most frequent bone tumor that predominantly targets the adolescent aged between 10-25 years. In the veterinary oncology, osteosarcoma accounts 80-95% of the bone tumors diagnosed in dogs. Humans and dogs share several similarities in regard to the physiological and molecular aspects of osteosarcoma development. For this reason dogs have been used as a homologous model to human, which have been showed promising in vitro results. Herein, from implanted tumor in nude mice we analyzed the effects of a combination of stem cells from canine bone marrow and bone morphogenetic protein (BMP-2) on the treatment of osteosarcoma. The results showed that this combination changed the expression of markers of cell death, leading to an increase of caspase 3 expression, and decreased of Bcl-2 expression, as well as decreased in cell proliferation (Ki-67 and p53) and receptor of angiogenesis pathways (CD34, COX-2, IL-6, IL-8, and VEGF). The tumor environment was modified by decreased expression of CD4 + CD25 + resulting in increased CD8 + cytotoxic. In conclusion, these data showed that the treatment using the combination of stem cells from canine bone marrow and bone morphogenetic protein (BMP-2) emerges as a potential therapeutic tool for the osteosarcoma treatment.

Keywords: Bone tumor; Morphogenetic proteins; Stem cells; Mesenchymal

Introduction

In humans, the osteosarcoma occurs mainly during the quickly bone growth, reaching 75-80% of the cases in individuals aged between 10 – 25 years [1-3]. In the veterinary oncology, osteosarcoma accounts for 80% to 95% of the bone tumors diagnosed in dogs [4]. Due the similar characteristics in several aspects with the human osteosarcoma as the predilection of sex, elevated body size, bone location, involvement of the bone metaphyseal region, aspect and histological classification, tumor aggressiveness, high probability of metastasis, response to chemotherapy, and prognosis [1,5]. The canine osteosarcoma have been used as a homologous model to human, which have been showed promising *in vitro* and *in vivo* results [1,6-8] .

The high incidence of metastasis (80-90%) is a challenge for medicine today [9]. These metastases occur mainly in distant places from the primary neoplasia [10]. The development of new blood vessels (angiogenesis) is a critical indicator of tumor growth and metastatic dissemination [11]. The VEGF expression correlates with the tumor progression and has proven to be a key indicator of prognosis in many malignant tumors and development of drugs [12,13]. The VEGF is a highly potent homodimeric protein that stimulates angiogenesis [13], which is vital to inducing the proliferation of endothelial cells and increasing vessel permeability [14].

Another point that needs to be consider is the role and contribution that mesenchymal stem cells can do in the oncology once these cells have been investigated mainly focusing in its role on imunomodulatory response which have been reported for adhesion and transmigration cascades similar to leukocytes [15-17].

Human bone marrow-derived mesenchymal stem cells (hMSC) as recently suggested by Abdallah et al. [18] as a group of clonogenic cells found in the bone marrow stroma that are capable of differentiation into several mesodermal cell lines.

Studies have indicated that bone morphogenetic proteins (BMPs) are a group of extracellular factors of the TGF-β superfamily which are known for their ability to induce bone formation [19] and also have anti-tumor effects. These proteins are able to inhibit the cell cycle by stimulating p21 (cyclin-dependent kinase inhibitor 1) overexpression [20,21]. Whereas Hardwick et al. [21] reported that the loss of the BMP signaling pathways led to increased tumorigenesis through a reduction in the apoptosis and cell adhesion.

Although several pathways have been investigated in canines [22], few molecules have been identified as prognostic tools or potential therapeutic targets [23]. Our group previously demonstrated that *in vitro* assays the BMP-2 associated with mesenchymal stem cells suppress the growth of the canine osteosarcoma cells [8].

Herein, we analyzed the cellular response to treatment with the association of mesenchymal stem cells with rhBMP-2 in order to investigate the cell proliferation, cell death, and angiogenesis pathways on canine osteosarcoma cells.

Material and Methods

Cell preparation

Osteosarcoma cell lines (OST) approved by Ethic Committee in the use of animals, protocol number 1654/2009.and undifferentiated mesenchymal stem cells from the bone marrow of canine fetuses (MSC),

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Received June 25, 2013; **Accepted** August 19, 2013; **Published** August 21, 2013

Citation: Rici REG, Will SEA, Favaron PO, Fratini P, Miglino MA, et al. (2013) Modulation of Angiogenesis and Immune Response in Canine Osteosarcoma by BMP-2 and Mesenchymal Stem Cells. J Stem Cell Res Ther 3: 143. doi:10.4172/2157-7633.1000143

approved by Ethic Committee in the use of animals, protocol number 931/2006, and undifferentiated mesenchymal stem cells from the bone marrow of canine fetuses (MSC) were obtained from the cell bank of the Stem Cells Laboratory of the Anatomy of Domestic and Wild Animals division, School of Veterinary Medicine and Animal Science, University of São Paulo and from the Laboratory of Biochemistry and Biophysics of Butantan Institute, São Paulo, Brazil.

Cell thawing

The osteosarcoma and mesenchymal bone marrow cell lines from canine fetuses were rapidly thawed in a water bath at 37°C. The pellet was centrifuged to remove the culture medium. Two washes were subsequently performed in PBS (phosphate buffer solution) in a centrifuge at 24°C and 1000 rpm for five minutes. The cell pellet was then resuspended in saline solution.

MSC and OST cell growth

The cells were maintained in 25 cm^2 culture flasks using DMEM-H (LGC) cell culture media supplemented with 10% fetal bovine serum (VITROCELL, Campinas, SP), 1% antibiotics penicillin and streptomycin (GIBCO) and 1% pyruvic acid (GIBCO) at pH=7.4 in a humidified incubator at 37°C with 5% CO_2. The cells were grown in a monolayer adherent to the culture dish surface. Following 72 hours of adherence and confluency, the OST and MSC cells were trypsinized, centrifuged and resuspended in PBS for application in the experimental animals. Trypan blue dye and a Neubauer chamber were used to count the cells and assess their viability, and a 1×10^6 concentration was prepared for the treatment. The MSC cells were split into two groups one of which was treated with a 5 nM rhBMP2 concentration as described by Rici REG et al. [8].

Cell transfection

The OST cells were transduced with LeGO IC2 lentiviral vector. The cells were plated (10^5 cells/well) 24 h prior to use. The next day, the plated cells were transduced with 10^7 viral particles per mL culture media with polybrene (1 µL per mL) and maintained on minimal medium for 4 h. Up to 3 mL of cell media was then added, and the cells were maintained in culture for 48 h. The cells were then trypsinized, centrifuged and applied to the experimental animals. The labeling efficiency of the transfected cell LEGO IC2 TMB was assessed after 2 days of growth in culture by flow cytometry for fluorescence microscopy demonstrating a positive above 70% of positive staining.

Bioluminescence protocol

X-ray images of the mice were acquired using an exposure time of 240 secondes, a 180 mm field of view and an f-stop of 2.80. The X-rays were acquired using an In-Vivo X-ray Imaging System (Carestream) with Carestream Molecular imaging software version 5.0.7.24 (Carestream, Woodbridge, CT, USA). The images were reconstructed using the respective 2-D ordered subsets expectation maximum (OSEM) algorithm. No correction was necessary for attenuation or spreading. The scanner had computer-controlled vertical and horizontal movements with an effective axial field of view (FOV) of 7.8 cm and a transaxial FOV of 10 cm. The mice were placed in the center of the field of view to provide a higher resolution micro-PET and greater sensitivity. The images were acquired with a 4 min scan using routine image acquisition scanning parameters.

Experimental treatment design

The 27 female BALB/$c^{nu/nu}$ mice, with an approximate weight of 22 g each and age of 6–8 weeks, received 10^6 transduced canine OST cells, applied dorsally under sterile conditions subcutaneously according to the protocol from the committee on the use of experimental animals of the School of Animal Science and Food Engineering, University of São Paulo, Pirassununga, Brazil. Thirty days after applying the OST cells, the mice were randomly separated into three groups for treatment with MSC and MSC combined with rhBMP-2 (B355510U6) Sigma, Gillinghan, UK) (Figure 1). The animals were kept in microisolator cages in ventilated racks located at the Butantan Institute Animal House.

Protocol for euthanasia

The mice were placed in a CO_2 chamber with a flow of 30% oxygen; a small quantity of CO_2 was injected for 30 s to reduce the anxiety that results from the discomfort of hypoxia. The CO_2 flow rate was increased to 3–4 times the initial flow rate to accelerate the process after the animals became unconscious. After the apparent clinical death of the animal, as determined by inspection of the eyes for fixed eyes and dilated pupils, the gas flow was maintained for a few seconds. Death occurred due to respiratory arrest after 2–5 min.

Flow cytometry analysis

The lymph nodes were macerated and filtered through a 30-µm membrane to prepare a cell suspension. The cells were resuspended in FACS (fluorescence-activated cell sorting) buffer, and the concentration was adjusted to 10^6 cells/mL. For intracytoplasmic and nuclear markers, the cells were permeabilized with 5 µl 0.1% Triton X-100 for 30 minutes prior to incubation with specific primary antibodies in a concentration of 1:100 for p53 (cellular tumor antigen p53) (ab-26), Ki67 (antigen identified by monoclonal antibody Ki-67) (ab-15580), CD4 (cluster of differentiation 4) (ab25475), CD8 (cluster of differentiation 8) (ab22378), cytochrome c (ab13575), Cox-2 (cyclooxygenase-2) (ab23672), IL-1 (interleukin-1) (ab7632), IL-8 (interleukin-8) (ab89336), IL-6 (interleukin-6) (ab6672), CD3 (cluster of differentiation 3) (ab113628), CD25 (cluster of differentiation 25), OCT3/4 (organic cation transporters 3 or 4) (ab18976), NANOG (Homeobox transcription factor NANOG) (ab80892), CD34 (cluster of differentiation 34) (ab8158) marking the extent of apoptosis and necrosis by annexin V (ab14196) (Abcam, Cambridge, USA), caspase-3 (sc7272), BAX (Bcl-2-associated X protein) (sc7480), Bcl-2 (B-cell lymphoma 2) (sc7382) (Santa Cruz Biotechnology, California, USA) with propidium iodide (PI) (81845) staining, the mitochondrial electrical potential using rhodamine 123 (r8004) (Sigma, Gillinghan, UK) and VEGF (clone VG1) (DakoCytomation, Carpinteria, CA, USA). The tubes were centrifuged and the supernatant discarded; the pellet was resuspended in 100 µL, and 1 µL of secondary antibody was added (ALEXA fluor 488 Molecular-Probe, mouse anti-goat IgG-FITC). The analysis was performed using a flow cytometer (FACSCalibur, BD), and the expression of the markers was determined by comparison with the control isotype.

Immunohistochemistry

Tumoral samples from group III were subject to immunohistochemistry for vascular endothelium growth factor - VEGF (1:300, Santa Cruz Biotechnology, Santa Cruz, California, USA), and p53 (1:50, Santa Cruz Biotechnology, Santa Cruz, California, USA). The negative control was performed using IgG (Goat anti-Mouse IgG, AP 308F, Chemical International). The histological sections were deparaffinized, and the endogenous peroxidase was

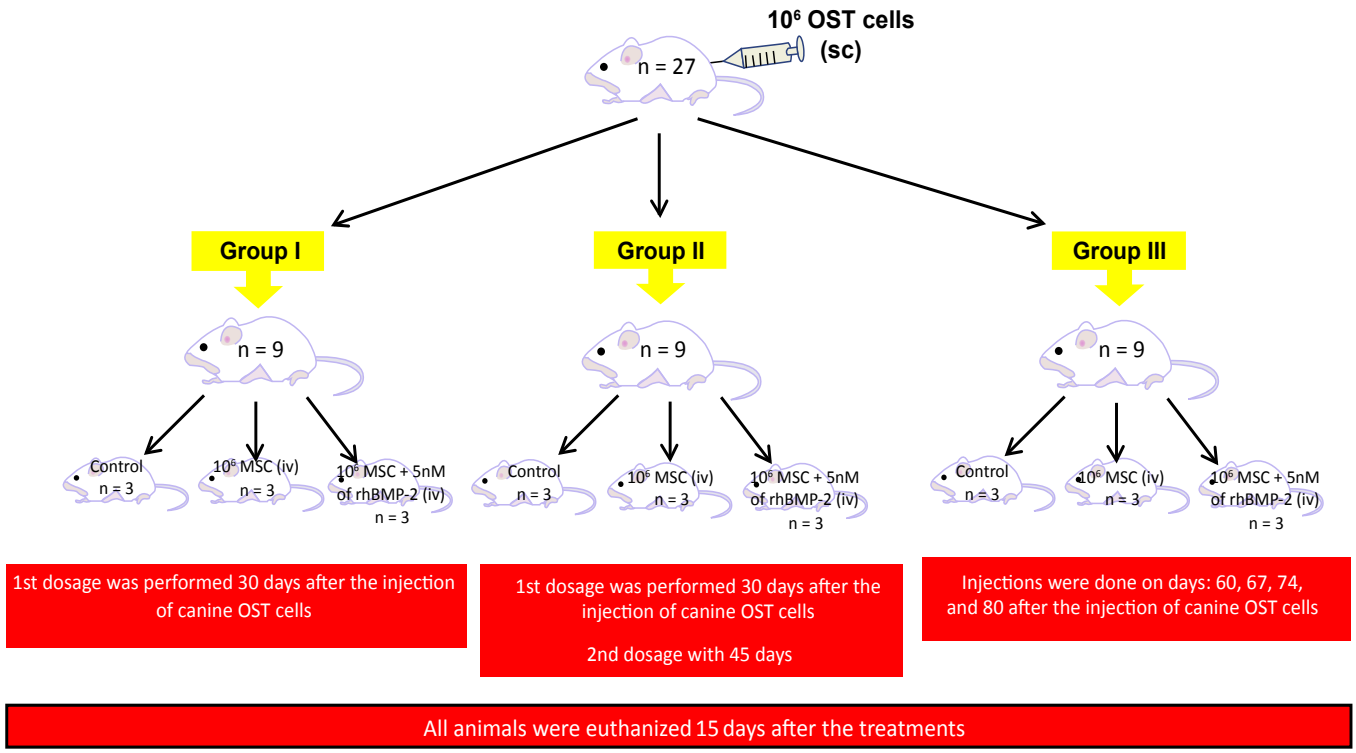

Figure 1: Experimental Treatment Design. Totally 27 female BALB/c$^{nu/nu}$ mice received 10^6 transduced canine OST cells applied subcutaneously (sc). Thirty days after applying the OST cells, the mice were randomly separated into three groups (I, II, and III) for treatment with MSC and MSC combined with rhBMP-2 which were injected in different periods.

subsequently blocked in 3% H_2O_2 in 100% ethanol, for 20 min. These sections were then hydrated in decreasing concentrations of ethanol, treated with 0.1 M citrate buffer at pH 6.0, and irradiated in a microwave oven (domestic model) at its maximum power (700 MHz) three times for 5 min. each. The sections were then equilibrated in 0.1 M phosphate buffered saline (PBS) at pH 7.4 wherein the protein blocking was performed using the Dako Protein Block kit (X 0909, DakoCytomation, Carpinteria, CA, USA) for 20 min. The samples were incubated with the primary antibody in a humidified incubator at 3°C for 12 hrs. After incubation, the sections were washed and incubated again with secondary antibodies conjugated using a peroxidase-based Dako LSAB kit (K 0690, DakoCytomation, Carpinteria, CA, USA) for 15 min, followed by streptoavidin from the same kit for 15 min. The reaction was visualized by the addition of a DAB developer (liquid DAB developer + substrate chromogen system, Dako Cytomation, Carpinteria, CA, USA) for 5 min. The sections were then counter-stained with hematoxylin and mounted using Faramount aqueous mounting medium (DakoCytomation, Carpinteria, CA, USA).

Statistical analysis

Data are expressed as mean ± SD, unless otherwise noted. Statistics were performed by using GraphPad Prism (v. 5), Microsoft Excel (v. 14), and the significance was accepted at $p<0.05$, unless otherwise noted.

Results

After 30 days of the injection of OST cells in the animals from group I the treatment started with the injection of MSC and MSC combined with rhBMP-2. Then, after 15 days the animals were euthanized and subjected to pathological analysis. During the pathological analysis there was not tumor dorsal and metastasis formation in this group. In the group II, which received two dosages of MSC and MSC, combined with rhBMP-2 (the first 30 days and the second 45 days) do not developed tumor dorsal and metastasis formation. The lymph nodes were collected from animals to evaluate in the cellular level the expression of markers for cell proliferation, immune response, angiogenesis, and cell death. Only in animals from group III after 60 days of OST cells was macroscopically observed the development of tumor dorsal and from that the tumoral was done by bioluminescence (Figure 2A), immunohistochemistry analysis (Figures 2B-2G), the expression of markers by flow cytometry, and the effects of MSC and MSC combined with rhBMP-2.

The results indicated a decrease in p53 expression for the treatment with MSC combined with rhBMP-2, which demonstrated the antiproliferative effect of the treatment. The expression of p53 by immunohistochemistry was observed in tumor tissues (Figures 2B-2D) and lymph nodes (Figures 2E-2G). Conversely, due to p53 immunolocalization, the group treated with MSC combined with rhBMP-2 exhibited an apparent decrease in p53 expression in both tissues (Figure 2D and 2G). The combination of MSC with rhBMP-2 regulated the p53, whose expression significantly decreased ($p<0.001$) over that of the control group (Figure 2H). The treatment with MSC caused a decrease in p53 expression in the groups I and II ($p<0.05$) and III ($p<0.01$) (Figure 2H).

The expression of Ki-67 significantly decreased for the mice treated with MSC/rhBMP-2 in groups I and II ($p<0.001$), and group

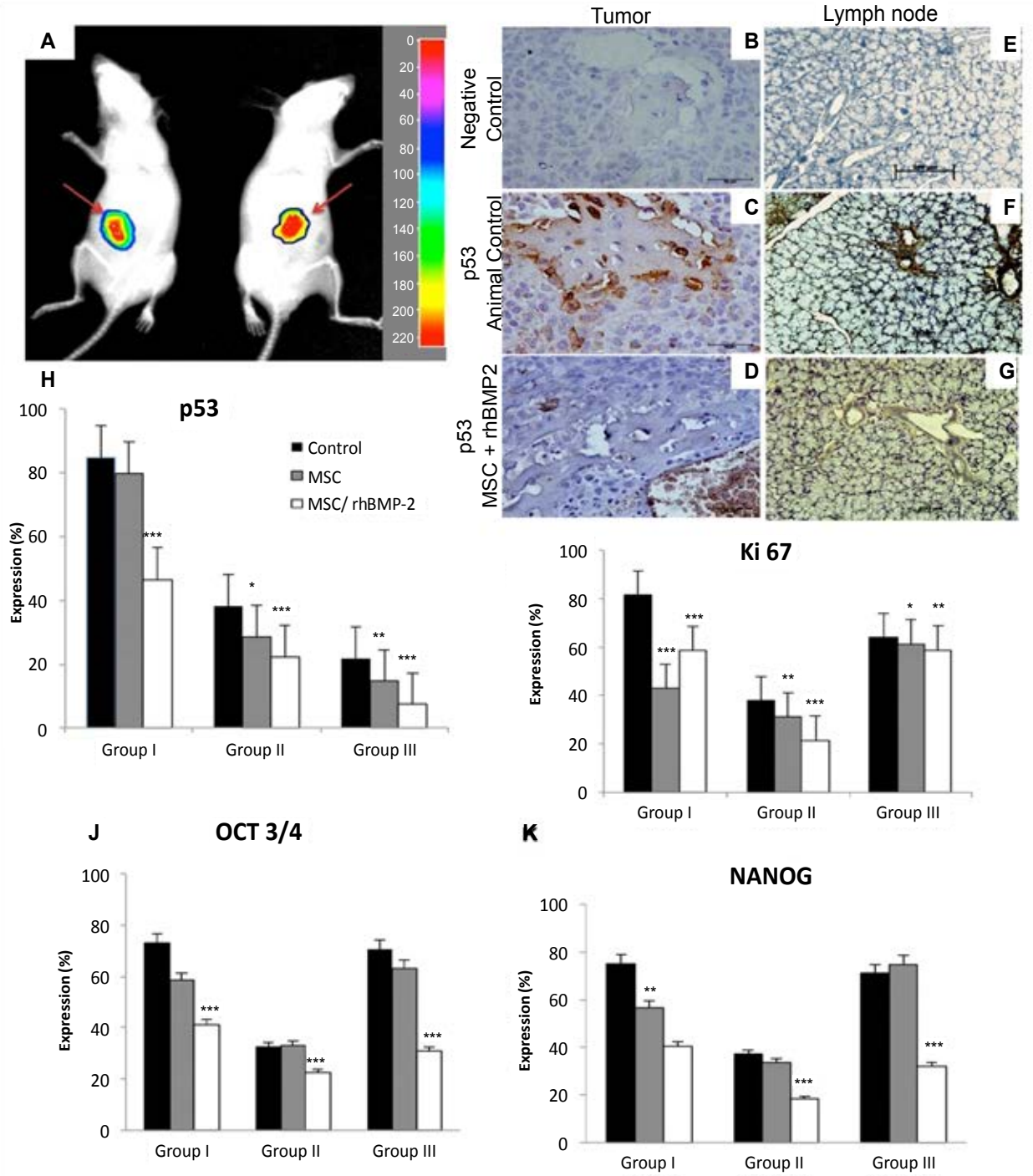

Figure 2: Expression of proliferation and pluripotency markers. (A): Identification by bioluminescence of the primary sites of OST cells (arrows) in the lombar region in animals from group III. OST cells were transduced with LeGO IC_2 lentiviral vector. (B-G): Immunohistochemestry for p53 on tumor and lymph nodes tissues from animals of the group III. In B and E: negative control. (C and F): animal control from group III. (D and G): tumor and lymph nodes tissue treated with MSC/rhBMP-2 showed a decreased expression of p53. (H-K): markers expression by flow cytometry treated with MSC, MSC/rhBMP-2. In (H): the most expressive reduction of the p53 expression occurred with the association of MSC/rhBMP-2. (I) expression of Ki67 reduced in the groups II and III. (J and K): the decreased expression of pluripotency markers (Oct ¾ and Nanog) was similar. Statistical differences were obtained by analysis of variance (*** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$).

III ($p<0.01$) over that of the control group. In addition, the cell proliferation expression showed a significant decrease after treatment with MSC in groups I ($p<0.001$) and II ($p<0.01$) and group III ($p<0.05$) (Figure 2I).

The OCT ¾ and NANOG for pluripotency showed a decreased expression in all treated groups. This decreased expression was significantly in all 3 groups treated with MSC/rhBMP-2 ($p<0.001$) (Figures 2J and 2K).

Tumoral formation and the development of metastasis in the intestine, lungs and spleen were detected in the control group via bioluminescence (Figures 3A, 3D and 3G). The points of metastasis in the intestine (Figure 3B), spleen (Figure 3E) and lungs (Figure 3H) in the animals from the control group were confirmed by necropsy. In contrast, metastasis was not observed in animals treated with MSC/

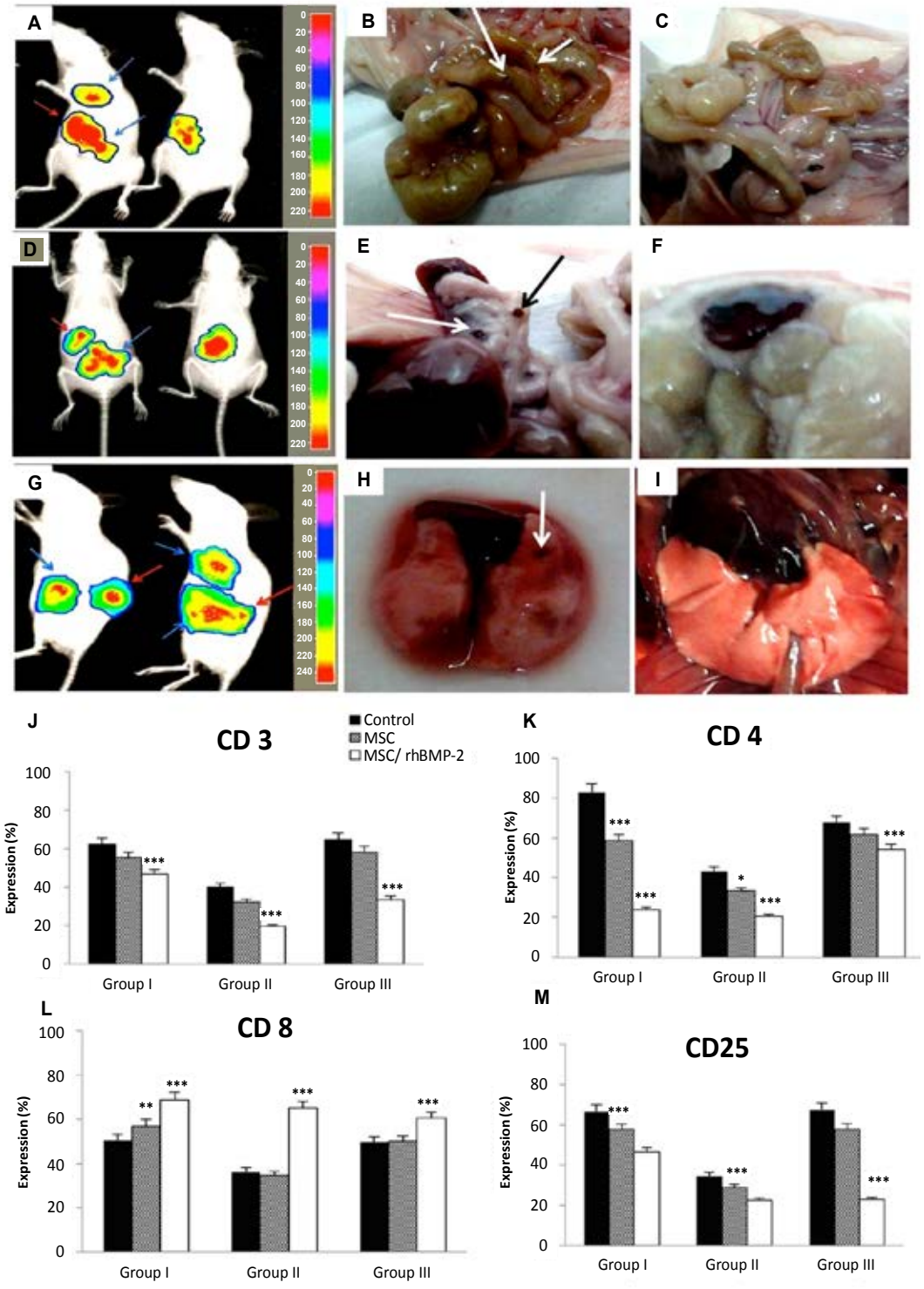

Figure 3: Expression lymphocytes and metastasis markers. Tumoral development was detected by bioluminescence (A, D, and G, arrows) and necropsy in the control animals of the group III. In: (B, E, and H): metastasis in intestine, spleen and lung, respectively in animals from group III (arrows). In (C, F, and I) no metastasis was observed in the same organs in animals from group III with association of MSC/rhBMP-2. In (J, K, and M) was observed a decreased in the expression of CD3, CD4, and CD25, respectively by flow cytometry in the analyzed groups. (L): In the groups with the association of MSC/rhBMP-2 there was an increased in the expression of CD8. Statistical differences were obtained by analysis of variance (*** $p < 0.001$, ** $p < 0.01$, and * $p< 0, 05$).

rhBMP-2 (Figures 3C, 3F and 3I). The expression of CD4, CD3, and CD25 in tissues from animals treated with MSC/rhBMP-2 showed a modulatory effect with a significant decrease in the expression ($p<0.001$) for all three groups (Figures 3J, 3K and 3M). A significant ($p<0.001$) increase was also observed in the CD8 (Figure 3L). The expression of all markers (except CD8) in the three treatment groups decreased with MSC. The CD4 expression was significant in groups I ($p<0.001$) and II ($p<0.05$) (Figure 3K). In contrast, the expression of CD25 was significant in groups I and III ($p<0.05$) (Figure 3M). The expression of CD8 in group I showed a significant increase ($p<0.01$) (Figure 3L).

In the three experimental groups there was a decreased expression of VEGF when the animals were treated with the association of MSC/rhBMP-2 ($p<0.001$) (Figure 4A). Macroscopically, when the control animals from group III were analyzed showed an intense vascularization surround the tumor (Figures 4B and 4C). When treated

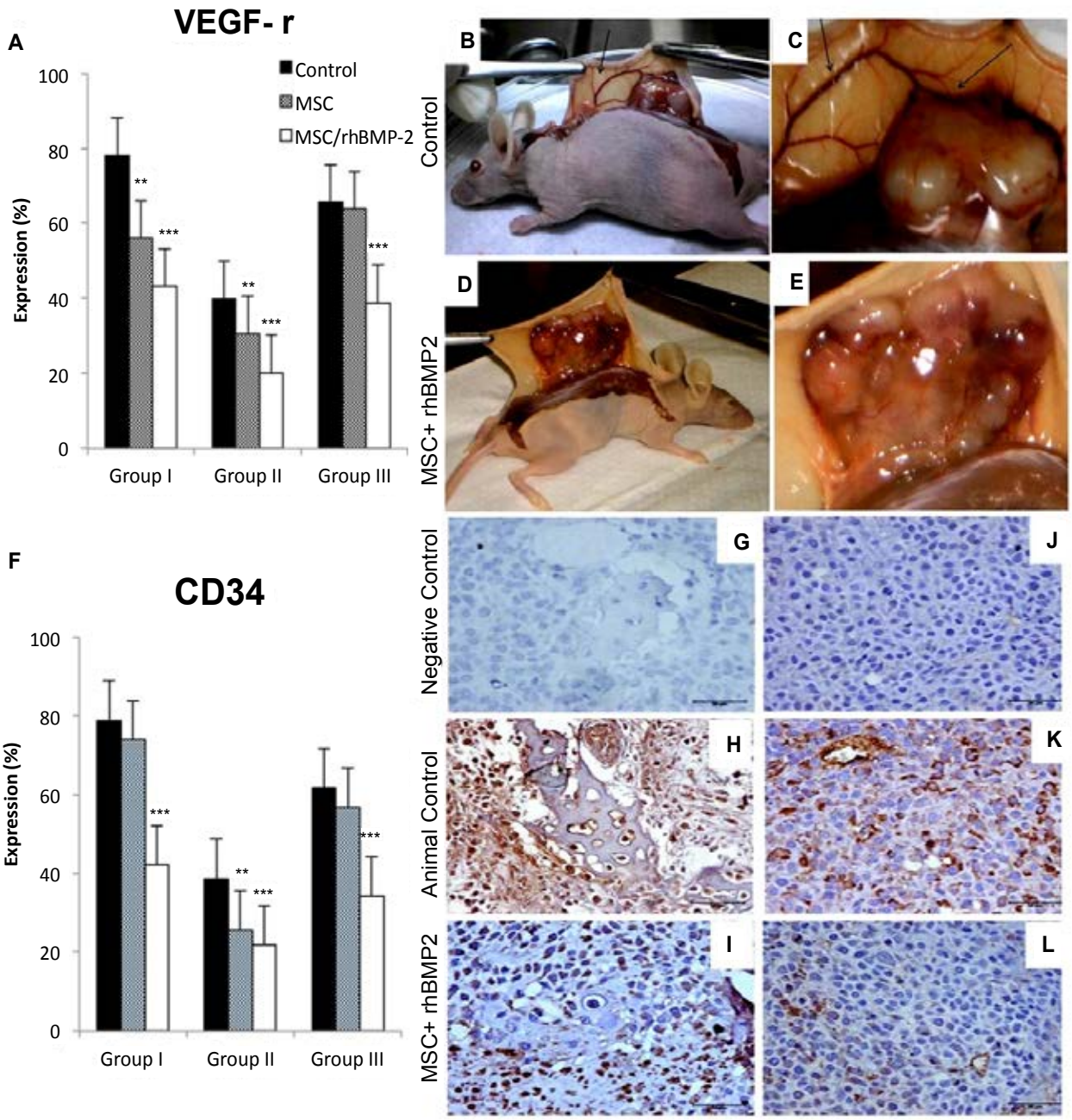

Figure 4: Expression of angiogenesis markers. In (A): expression of VEGF-r by flow cytometry in all analyzed groups. Observed that in all experimental groups there was a reduction using the association of MSC/rhBMP-2. In (B and C): vessels surrounding the tumor (arrows) in animals from group III. In contrast to the control animals the treatment using the association of MSC/rhBMP-2 showed an expressive reduction of the blood vessels related to the tumor in the group III. (F): expression of CD34 was reduced in all treatments. (G-L): immunohistochemestry for VEGF-r. In (G and J) negative controls for the antibody. In (H and K) expression of VEGF-r in tumoral tissue from control animals of the group III. In (I and L): a reduction in the VEGF-r expression was observed in the tumoral tissue from animals treated with the association of MSC/rhBMP-2 from the group III. Statistical differences were obtained by analysis of variance (*** $p < 0.001$, ** $p < 0.01$, and * $p< 0, 05$).

with MSC/rhBMP-2, macroscopically was observed a reduction of the vessels around the tumor (Figures 4D and 4E).

The CD34 expression decreased significantly in the three groups treated with MSC/rhBMP-2 ($p<0.001$) (Figure 4F). As in flow cytometry analysis, immunohistochemistry for VEGF showed a reduction of expression in the group treated with MSC/rhBMP-2 (Figures 4I-4L) when compared with the control animals of the group III (Figures 4H-4K).

The expression of COX-2, IL-1, IL-6, and IL-8 decreased in the treated groups with MSC/rhBMP-2, when compared with the controls. The COX-2 expression decreased significantly in the group I ($p<0.001$) and in groups II and III ($p<0.01$) (Figure 5A). In the treated groups, IL-1 expression decreased significantly in groups I and II ($p<0.01$) and in group III ($p<0.05$) (Figure 5B). IL-6 expression decreased in groups I and II ($p<0.001$) and in group III ($p<0.05$) (Figure 5C). IL-8 expression decreased in groups I and III ($p<0.01$), but in group II, the decrease was not statistically significant (Figure 5D). The pathways involved in angiogenesis and metastasis with special attention in the participation of COX-2 and IL-8 in these functions is demonstrated in the Figure 5E.

The results indicated that a significant activation of caspase-3 occurred with the release of cytochrome c into the cytoplasm. There was a decreased expression of Bcl-2 and the increased expression of BAX in all experimental groups treated with MSC/rhBMP-2 (Figures 6A, 6B and 6C).

Analysis of the expression of Anexin V/PI showed a significantly increased of cell death by necrosis when treated with MSC/rhBMP-2 and by apoptosis in the group I, when treated with MSC ($p<0.001$) (Figure 6D). In the group II was observed in both treatments (MSC and MSC/rhBMP-2) that the cell death pathway occurred by necrosis ($p<0.001$) (Figure 6E). In the group III the cell death pathway occurred by apoptosis when treated with the association of MSC/rhBMP-2 ($p<0.001$), and when treated with MSC it occurred by necrosis (Figure 6F).

The ratio of necrotic cells to cells in late apoptosis increased significantly ($p<0.001$) in the MSC/rhBMP-2 treated mice (group I). Conversely, the animals from group II demonstrated an increase in cell necrosis; however, this increase was less significant ($p<0.001$) than that of the cells in late apoptosis ($p<0.05$). In contrast with the previously mentioned groups, death was induced through the apoptosis pathway in group III ($p<0.001$) (Figures 6D, 6E and 6F).

Functional alterations in the mitochondrial electrical potential were studied using the rhodamine 123 probe, which showed that the intrinsic pathway of apoptosis promoted a decrease in the mitochondrial function for all three groups treated with MSC/rhBMP-2 ($p<0.001$) (Figures 6G and 6H).

Discussion

Dogs and human share several similarities in relation to the physiology. For this reason, dogs have been used as animal model for several purposes, including for provide an important model of human cancer biology and cancer therapeutic strategies [24]. In this way, the study of canine osteosarcoma can provide a deeper understanding of human osteosarcoma. In this current research we establish a treatment using a combination of BMP-2 and mesenchymal stem cells in order to investigate the effects of that on canine osteosarcoma cells.

The differences that were found among the control groups I, II and III were related to the kinetic changes on the tumor growth that was followed by the presence of fibrotic and necrotic areas, which were responsible for the tissue remodeling and induction of an intratumoral inflammatory response. This data was supported by the changes in the expression of p53 and ki67, as a proliferative marker. The results obtained with the control groups (I, II and III) is in accordance with Vesely et al. [25] which divided the immunomodulation of the cancer antitumoral responses in three phases: elimination phase, which the innate and adaptive immunity that work together in order to eliminate the early tumors during the initial stage of tumor development. Then, the equilibrium phase, in which is present only tumor cells resistant to antitumor mechanisms of the innate system [26]. In the last phase, exhaust phase, the tumor is out of control and release a lot of tumorigenic cells with results in metastasis. Other important point is the osteosarcoma is immunologically "quiet", i.e. there is negligibe presence or inflammatory cells in the tumor mass [27]. Therefore, introduction of immonomodulators as the MSC and BMP-2 combination therapy activate immune cells and increase their infiltration into tumors.

The results showed a decrease in the p53 expression during the treatment with MSC combined with rhBMP-2 resulting in an antiproliferative effect during the treatment. In the groups I, II and III, the rhBMP-2 regulated the activity of p53. The p53 stimulates the transcription of several genes in order to mediate its two main effects: cell cycle arrest and apoptosis, through binding with DNA [28]. Studies on Hodgkin's lymphoma showed via staining that the mutation of the p53 gene may play a key role in tumor development [29]. The strong staining in high-grade lymphomas suggests that p53 may be involved in the transformation of a low-grade lymphoma into a high-grade lymphoma. Thus, p53 immunolocalization in lymphoid lesions may be indicative of their malignancy [30]. In addition, our results showed that there was in group III (treated with MSC/rhBMP-2) a decrease in the expression of p53 in tumor and lymph node tissues. Bongiovanni et al. [31] demonstrated that the high levels of p53 nuclear expression increased significantly with the development of metastases. Thus, changes in the roles played by p53 are linked to tumor behavior [32]. Researches on Ki-67 proved a viable marker for measuring cell growth in human neoplasms [33]. Hernandez-Rodriguez et al. [34] demonstrated that Ki-67 nuclear staining was linked to an increase in lung metastases and tumor mortality in over 50% of the tumor cells in a primary osteosarcoma. The Ki-67 overexpression was also demonstrated by Hernandez-Rodrigues et al. [34] in patients who developed lung metastases. Weiming et al. [35] demonstrated a Ki-67 correlation between the tumor size and degree of differentiation and the proliferative capacity and early metastases. in contrast with the control group and that treated with MSC only, our results indicated a decreased expression of the Ki-67 marker in the three groups treated with MSC combined with rhBMP-2, suggesting that this combination can regulate osteosarcoma cell proliferation. According to Peña et al. [36], the increased levels of the Ki-67 marker have a positive correlation with metastasis, cancer death and a shorter survival time. Marinho et al. [37] demonstrated that Ki-67 can be used as an indicator of prognosis in osteosarcoma patients both with and without metastasis upon diagnosis. Studies have demonstrated the role of Oct3/4, NANOG and Sox-2 in tumorigenesis [38,39]. According to Giedekel et al. [40], the Oct3/4 acts as a multifunctional factor in cancer biology increasing the malignant potencial of embryonic stem cells in a dose-dependent manner, which is a possible oncogenic role attributed to Oct ¾. In addition, the Oct3/4 performs a critical role for the tumor growth [41-43]. Studies have shown that the Oct3/4 gene is expressed in some human tumor cells, but not in normal somatic cells [44]. In

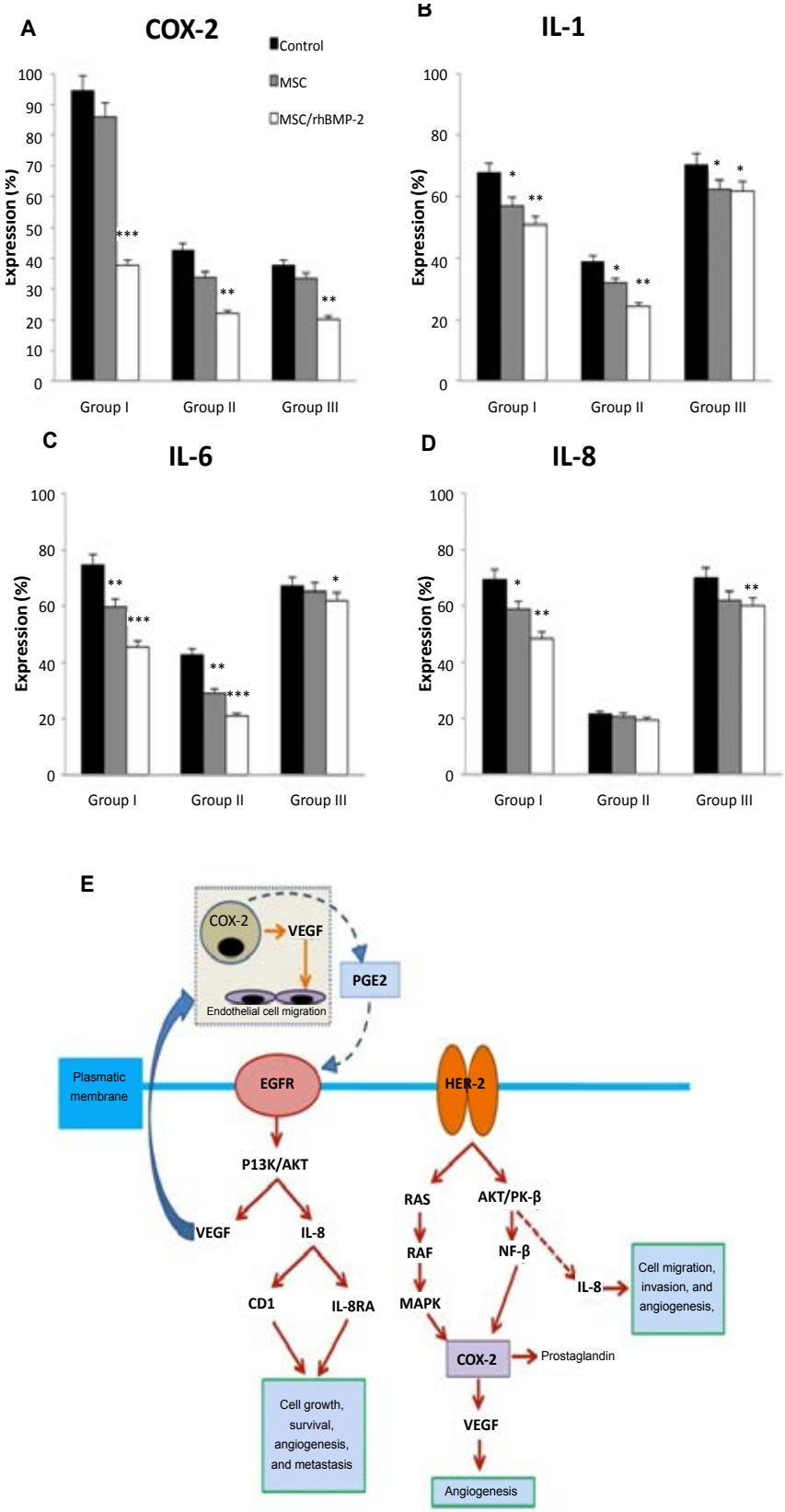

Figure 5: Pro- and anti-inflammatory expression of receptor markers. In (A, B, C and D) the expression of COX-2, IL-1, IL-6, and IL-8 activity decreased in the MSC/rhBMP-2 treatments when compared with the controls. In (E): Representative scheme of the pathways involved in the COX-2 and IL-8 signaling. Statistical differences were obtained by analysis of variance (*** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$).

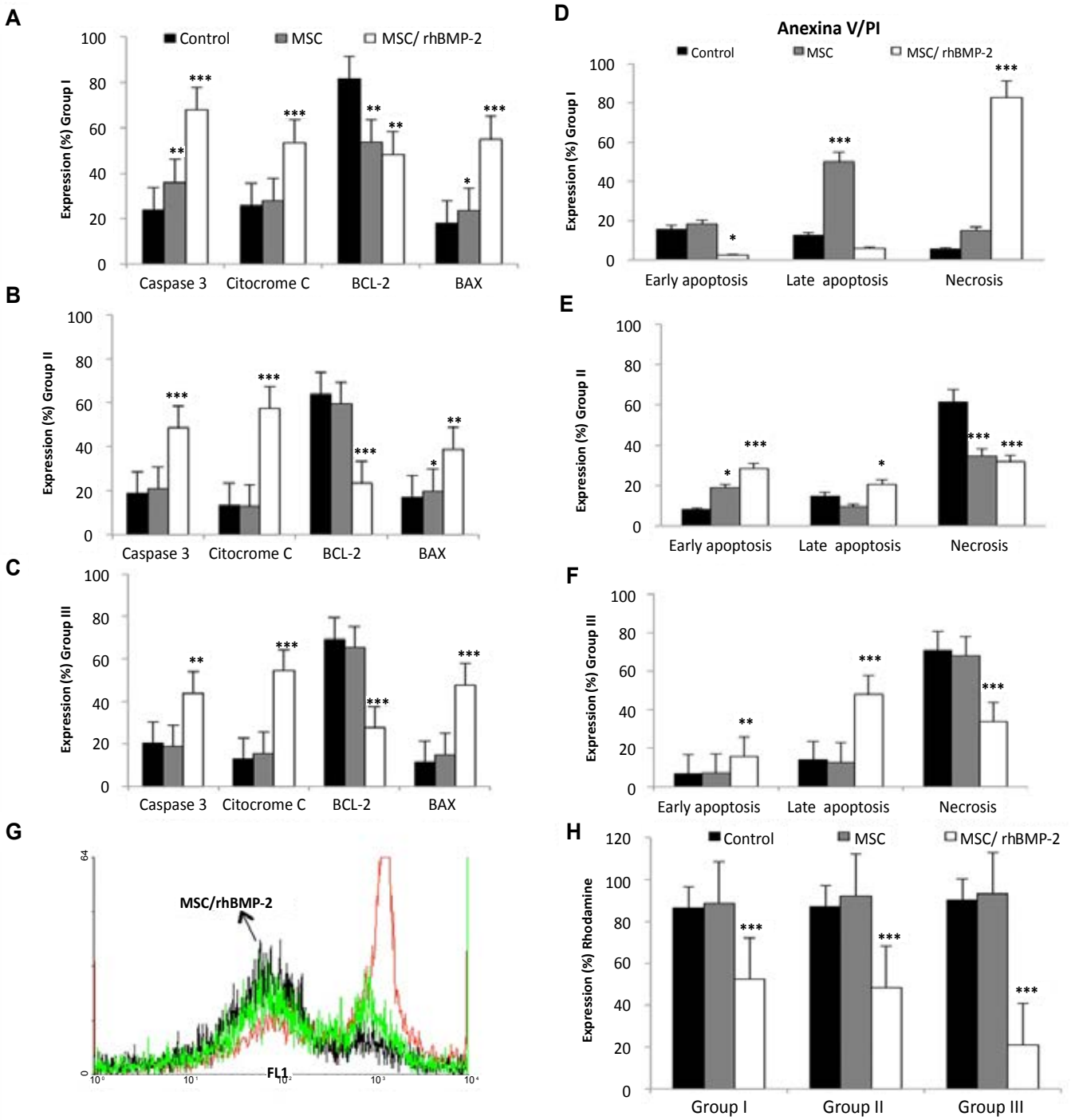

Figure 6: Expression of markers for cell death pathways. In (A, B, and C) significantly activation of caspase-3 with cytochrome c, and BAX in all treated groups with MSC/rhBMP-2. There was a decreased in the BCL-2 expression in all treated groups with MSC/rhBMP-2. In (D): Observed an increased in the expression of markers for cell necrosis in the group I treated with MSC/rhBMP-2 and late apoptosis treatment MSC. In (E): variation in expression of anexina V/PI was observed in all treatments on group II, but was observed a prevalecence of death by necrosis. In (F): In the MSC treatment the death pathway was by necrosis with a prevalecence of apoptosis pathway in animals treated MSC/rhBMP-2 on the group III. In (G and H): A reduction of expression of mitochondrial electrical potential was observed in all groups treated with MSC/rhBMP-2. Statistical differences were obtained by analysis of variance (*** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$).

our experiments, the Oct3/4 and NANOG decreased significantly in all treated groups, which were more pronounced in the group III, where the animals were treated with MSC/rhBMP-2.

Evidence indicates that the tumors use mechanisms that suppress the immune system to prevent host immunity [45,46]. These mechanisms include components of the host immune system, CD4+ CD25+ regulatory T cells, myeloid suppressor cells, and natural killer T cells (NKT) [47]. An increase in the mean percentage of infiltrating CD4+ T lymphocytes was noted in patients with lymph node

metastasis, but not CD8+ T cells. The relationship between the tumor stage and the T lymphocyte infiltration has been shown in individuals with prostate, gallbladder, kidney, colorectal and breast cancer [47]. A population of CD4+ T cells, which are referred as Treg (regulatory T) cells, is known to inhibit cytotoxic T cell responses to specific antigens [48]. The occurrence of T lymphocytes in human osteosarcoma was previously studied via immunohistochemical staining. The infiltration of CD3+ lymphocytes is correlated with malignancy and the occurrence of metastasis upon diagnosis. Phenotypic analysis indicated that these infiltrating lymphocytes in osteosarcoma were 95% CD3+ and 68% CD8+ [49]. The animals treated with MSC/rhBMP-2 showed a modulating effect with decreased expression of the CD4+ and CD25+ in all three study groups and an increased expression of CD8+. Rivoltini et al. [50] demonstrated the predominance of CD8+ by analyzing the phenotype of TIL (tumor-infiltrating lymphocytes) in 37 pediatric tumors including 12 osteosarcomas. The association between the CD4+ and CD25+ cells can maintain the tumor environment and reduce the tumor immunogenicity, thus allowing the progressive growth of tumors by blocking the action of cytotoxic CD8+ cells [51,52].

Tumor-infiltrating lymphocytes (TILs) are able to synthesize and release vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), which can induce angiogenesis and lymphangiogenesis. Thus, TILs could allow the dissemination of cancer cells through lymphatic vessels into regional lymph nodes [53]. During the initial stages of the osteosarcoma invasion, growth factors such as TGF-β are released from the bone matrix to act on the osteosarcoma cells, stimulating the release of interleukin-6 (IL-6) and interleukin-11 (IL-11) [54]. Under hypoxia and acidosis conditions the osteosarcoma cells release endothelin-1 (ET-1), VEGF and PDGF (platelet-derived growth factor) [55]. Recently, Holzer et al. [56] demonstrated for the first time the direct connection between a VEGF high concentration and malignant osteosarcoma *in vivo*. Our results showed a decrease in the expression of both VEGF and CD34 markers in all experimental groups upon treatment with MSC+rhBMP2. The increased synthesis of VEGF has been shown to affect the growth of several solid tumors, including gastric, esophageal, colorectal, kidney, lung and breast osteosarcomas, and carcinomas in humans [57]. Studies also indicated that VEGF-positive osteosarcoma patients have worse survival rates than those with VEGF-negative tumors [14]. Oda et al. [58] also used VEGF immunostaining to prove that VEGF is regulated in osteosarcomas and is linked to metastasis, such that the individual survival rate decreased with increasing VEGF. Furthermore, data found in clinicopathological patients suggest that VEGF is expressed in malignant and cartilaginous tumors so that increased cellular levels are correlated with high pathological grades [59].

Tumor-associated macrophages (TAMs) can release a variety of toxic agents into the tumor microenvironment including hydrogen peroxide (H_2O_2), proteases, immunosuppressive substances and several growth factors and cytokines (endothelial growth factor EGF, vascular endothelial growth factor VEGF, macrophage colony-stimulating factor M-CSF, IL-1, IL-8) and, consequently, may lead to increased mitotic activity and the establishment of an immunosuppressant network that promotes tumor growth, invasion and neoangiogenesis [60]. Our results indicate a decreased expression of the IL-1, IL-6, and IL-8 and COX-2 receptors in the MSC/rhBMP-2-treated groups, suggesting an inhibition of the local inflammatory response. According to Wierenga et al. [61], JAK protein degradation can interfere with the IL-6 signaling. Treatment with 4 doses of MSC/rhBMP-2 (Group III) showed a significant decrease in expression of IL-1, IL-8 and IL-6 receptors, with no significant quantitative changes in the values of COX-2. Several studies have shown that cytokines can modulate tumor development [62]. In this regard, IL-1 appears to block the action of the appetite stimulator neuropeptide-Y, in the central nervous system. The TNF-α and IL-1 receptors have been found in areas of the hypothalamus that regulate hunger, and an IL-1 infusion in healthy mice was able to alter food intake and the number and volume of meals [63]. Macrophage activation is primarily characterized by the secretion of IL-1, IL-6, and TNF-α and by the secretion of reactive oxygen and nitrogen species with increased phagocytic capacity [64]. Chow et al. [65] demonstrated that more than 50 genes were individually expressed in tumor-associated macrophages (TAMs) when compared to the controls, including IL-6, IL-8 and MMP-9 (metalloproteinase-9).

Fujimoto et al. [66] demonstrated the IL-8 effect on the migration of tumor cells and their involvement in cervical carcinoma angiogenesis, in combination with the vascular endothelial (VEGF) and platelet-derived (PDGF) growth factors produced by malignant cells or stroma. The positive correlation between the number of infiltrating macrophages, the degree of vascularization and the disease prognosis was demonstrated previously at the tumor site in patients with breast cancer [67]. As shown in the present study, the expression of VEGF and IL-8 decreased. Interleukin-8 has also been presented as a powerful angiogenesis promoter [46]. The ability of IL-8 to promote angiogenesis, a resistance to chemotherapy and the survival of tumor cells, makes it a powerful modulator for tumor development [68].

Studies have indicated that BMP-2 can decrease AKT activity and increase the caspase-3, 8, and 9 activities in smooth muscle cells under hypoxic conditions in a dose-dependent manner [69]. Zhang et al. [70] showed that the increase in caspase activity was mediated by the decrease in Bcl-2. Our results demonstrated significant caspase-3 activation with cytochrome c release into the cytoplasm with decreased anti-apoptotic protein Bcl-2 and increased pro-apoptotic BAX expression levels in all groups under the given experimental conditions using MSC/rhBMP-2. The Bcl-2 is known to be expressed at high levels in osteosarcomas and plays a role in the modulation and decrease of BAX gene expression through the induction of apoptosis and the suppression of the growth of tumor cells including osteosarcoma [71-72]. Therefore, an increased Bcl-2 expression can contribute to the intrinsic osteosarcoma chemoresistance, and may provide a promising target for gene therapy [73].

Zhao et al. [74] also demonstrated that a reduction in the Bcl-2/BAX ratio is linked with an induction of apoptosis and cell growth suppression in many tumors. A significant increase in the number of necrotic cells was noted in our study, especially in the MSC/rhBMP-2-treated group (group I). Animals from group II also showed an increase in the number of necrotic cells, but to a lesser extent. Conversely, the animals from Group III showed an induction of cell death via apoptosis and increased late apoptosis, demonstrating the efficacy of MSC/rhBMP-2 in controlling OST growth.

The importance of VEGF during the tumor metastasis has been indicated by the correlation between its expression in the primary tumor and the metastic rates, as well as the poor prognosis [75]. The VEGF in some osteosarcome and its expression greater than 30% of tumor cells has been associated with a poor outcome [76]. Herein, we demonstrated how is efficient the therapeutic response of the MSC associated with BMP-2 according to the inhibition of both production and release of angiogenic factors together to the inflammatory response markers.

The association of MSC/rhBMP-2 was effective in modulating

the growth, cell death, angiogenesis and cellular immune response in canine osteosarcoma. This treatment can be use as a new research tool and open new perspectives in the osteosarcoma treatment.

Conflict of Interest

The authors declare that they have no competing financial interests.

Acknowledgements

This research was supported by grants from CNPq, CAPES and FAPESP. In addition, we are grateful to the National Institute of Science and Technology in Stem Cell and Cell Therapy (INCTC). We thank Dayane Alcântara for the technical support.

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This article was originally published in a special issue, **Tissue Specific Stem Cells** handled by Editor(s). Dr. Morayma Reyes, University of Washington, USA